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In diagnostic imaging procedures, e.g. X-ray, MRI, ultrasound, light imaging and nuclear imaging, it has long been known to use contrast agents to facilitate visualization of particular organs or tissues or to identify diseased or malfunctioning regions, ie. generating morphological images.

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pH and temperature are particularly important as

indicators of abnormality or malfunction.

Several in vivo methods, both imaging techniques and non-imaging techniques, can be used to study physiological parameters, e.g. to diagnose disease. Typical non-imaging techniques include simple blood pressure measurements, electrocardiography or electrocephalography for detection of electric currents in the heart muscle and brain, respectively, and other simple tests performed in doctors' offices or hospitals. Today, a variety of imaging techniques are also used. The most frequently used methods include various X-ray based techniques, MRI, ultrasound and diagnostic methods based on radioactive materials (e.g. scintigraphy, PET and SPECT). Other diagnostic imaging methods include light imaging modalities, Overhauser MR (OMRI), oxygen imaging (OXI) which is based on OMRI, magnetic source imaging (MSI), applied potential tomography (APT) and imaging methods based on microwaves.

The images obtained in X-ray techniques reflect the different densities of structures/organs/tissues in the patient's body. Contrast agents are today used to improve the image contrast in soft tissue examinations. Examples of such contrast agents include gas (negative contrast effect relative to tissue); barium sulphate suspensions; and iodinated agents including ionic monomeric agents, non-ionic monomers, ionic dimers and non-ionic dimers. Typical examples of commercial X-ray contrast agents are Omnipaque[®] and Visipaque[®].

MRI is an imaging method generally based on interactions between radiowaves and body tissue water protons in a magnetic field. The contrast parameter or signal intensity is dependent on several factors including proton density, spin lattice (T_1) and spin spin (T_2) relaxation times of water protons. Typical commercial MRI contrast agents include Omniscan[®], Magnevist[®] and ProHance[®].

Ultrasound is another valuable modality in

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diagnostic imaging as it does not involve the use of ionizing radiation. In ultrasound examinations the patient is generally exposed to sound waves in the frequency of 1-10 MHz. These sound waves (or ultrasound waves) penetrate through or are reflected from the tissue. The transmitted or reflected sound waves are detected by a "microphone" and form the basis for development of a ultrasound image. Ultrasound imaging is a method of choice in pregnancy checks and birth control, and diagnosis of cardiovascular and liver diseases.

Although ultrasound contrast agents have been approved, there is as yet no general use of these agents. The main reason for this is the poor efficacy of the "first generation" agents. The ultrasound contrast agents currently under development are based on encapsulated gas because the reflection of sound from the liquid-gas interface is extremely efficient.

Typical ultrasound contrast agents are gas encapsulated in a sugar matrix, in a shell of denaturated albumin/or partly denaturated albumin, in polymers, and in surfactants including phospholipids. A typical ultrasound contrast agent with high contrast efficacy consists of a fluorinated gas bubble (for example SF₆ or a perfluorocarbon such as perfluoropropane or perfluorobutane) coated with a mono or multilayer phospholipid membrane. The particle size will generally be around 4 micrometer with very few particles larger than 10 micrometer in diameter. The main indications for such a typical product in the future may be cardiac imaging (cardiac perfusion examinations) and liver imaging.

Nuclear medicine imaging modalities are based upon administration of radioactive isotopes followed by detection of the isotopes, e.g. using gamma camera or positron emission tomography (PET). The most frequently used examination is gamma camera detection of 99-

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1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

Light imaging methods are performed using contrast agents that absorb and/or emit light (generally near infrared light).

APT based methods can also be performed (like for example thallium scans) without use of contrast agents; again however, contrast agents based on physiologically acceptable ions or other agents with effect on conductivity improve the diagnostic utility of APT.

However, there has been a great interest in measurement and quantification of various physiological parameters. (See for example J. Magn. Reson. Imaging 1997, 7, 82-90 for a review on physiologic measurements by contrast enhanced MR imaging).

Various methods for measurements of physiologically important parameters have been described in the scientific literature: tissue pH has been measured using near infrared reflectance spectroscopy (J. Clin. Monit. 1996, 12, 387-95); intratumor pH has been measured using ^{19}F magnetic resonance spectroscopy (Invest. Radiol. 1996, 31, 680-9); 6-fluoropyridoxal polymer conjugates have been suggested as ^{19}F pH indicators for magnetic resonance spectroscopy (Bioconjug. Chem. 1996, 7, 536-40); spectral imaging microscopy has been used for simultaneous measurements of intracellular pH and Ca^{2+} in insulin-secreting cells (Am. J. Physiol. 1996, 270, 1438-46); fluorescence ratio imaging has been used for measurement of interstitial pH in solid tumors (Br. J. Cancer 1996, 74, 1206-15); a fluorinated pH probe for ^{19}F magnetic resonance spectroscopy has been used for in

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cellular pH using ^1F NMR spectroscopy (FEBS Lett. 1994, 349, 234-8); lactate and pH have been mapped in calf muscles of rats during ischemia/reperfusion assessed by in vivo proton and phosphorus magnetic resonance chemical shift imaging (Invest. Radiol. 1994, 29, 217-23); nuclear magnetic resonance spectroscopy has been used for measurement of in vivo and ex vivo pH (Eur. J. Lab. Med. 1996, 4, 143-156); seminaphthofluorescein-calcein has been tested as fluorescent probe for determination of intracellular pH by simultaneous dual-emission imaging laser scanning confocal microscopy (J. Cell Physiol. 1995, 164, 9-16); ampholytic dyes have been proposed for spectroscopic determination of pH in electrofocusing (J. Chromatogr. A 1995, 695, 113-122); EPR spectroscopy has been used for direct and continuous determination of pH values in nontransparent water-in-oil systems (Eur. J. Pharm. Sci. 1995, 3, 21-6); intracellular Ca^{++} and pH have been imaged simultaneously in glomerular epithelial cells (Am. J. Physiol. Cell Physiol. 1993, 46, 216-230); fluorinated macromolecular probes have been evaluated for non-invasive assessment of pH by magnetic resonance spectroscopy (Bioorg. Med. Chem. Lett. 1993, 2, 187-192); pH has been mapped in living tissue by application of in vivo ^{31}P NMR chemical shift imaging (Magn. Res. Med. 1993, 29, 249-251); fluorescence spectroscopy has been used to measure temperature dependent aggregation of pH-sensitive phosphatidyl ethanolamine oleic acid-cholesterol liposomes (Anal. Biochem. 1992, 207, 109-113); ^{13}C NMR spectroscopy has been used to determine intracellular pH (Am. J. Physiol. Cell. Physiol. 1993, 264, C755-C760); ^{31}P NMR chemical shift imaging has been used for pH mapping of living tissue (Magn. Reson. Med. 1993, 29, 249-251); fluorescent probe and ^{31}P NMR spectroscopy have been compared for measurement of the intracellular pH of propionibacterium acnes (Can. J. Microbiol. 1993, 39, 180-6); panoramic imaging of brain pH and CBF has been

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Parameter	Unit	Value
Temperature	°C	25.0
Pressure	atm	1.0
Flow rate	L/min	1.0
Wavelength	nm	254
Scan rate	nm/min	20
Integration time	s	10
Resolution	nm	2
Slit width	nm	5
Beam diameter	mm	10
Sample thickness	mm	1.0
Sample concentration	mg/L	1.0
Sample volume	μL	10
Sample type		Water
Sample name		Water
Sample ID		Water
Sample location		Water
Sample date		Water
Sample time		Water
Sample operator		Water
Sample instrument		Water
Sample method		Water
Sample result		Water
Sample status		Water
Sample comment		Water
Sample notes		Water
Sample history		Water
Sample log		Water
Sample report		Water
Sample summary		Water
Sample details		Water
Sample analysis		Water
Sample interpretation		Water
Sample conclusion		Water
Sample recommendation		Water
Sample action		Water
Sample follow-up		Water
Sample review		Water
Sample approval		Water
Sample signature		Water
Sample stamp		Water
Sample seal		Water
Sample label		Water
Sample tag		Water
Sample marker		Water
Sample indicator		Water
Sample sensor		Water
Sample detector		Water
Sample actuator		Water
Sample controller		Water
Sample interface		Water
Sample module		Water
Sample component		Water
Sample part		Water
Sample piece		Water
Sample item		Water
Sample object		Water
Sample entity		Water
Sample instance		Water
Sample example		Water
Sample case		Water
Sample situation		Water
Sample scenario		Water
Sample event		Water
Sample occurrence		Water
Sample incident		Water
Sample accident		Water
Sample disaster		Water
Sample catastrophe		Water
Sample tragedy		Water
Sample misfortune		Water
Sample setback		Water
Sample reverse		Water
Sample decline		Water
Sample drop		Water
Sample fall		Water
Sample plunge		Water
Sample plummet		Water
Sample nosedive		Water
Sample crash		Water
Sample collapse		Water
Sample tumble		Water
Sample topple		Water
Sample tipple		Water
Sample tip		Water
Sample tilt		Water
Sample slant		Water
Sample slope		Water
Sample incline		Water
Sample ascend		Water
Sample climb		Water
Sample mount		Water
Sample rise		Water
Sample surge		Water
Sample boom		Water
Sample flourish		Water
Sample exult		Water
Sample triumph		Water
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Sample deed		Water
Sample act		Water
Sample action		Water
Sample activity		Water
Sample behavior		Water
Sample conduct		Water
Sample performance		Water
Sample display		Water
Sample show		Water
Sample exhibition		Water
Sample demonstration		Water
Sample presentation		Water
Sample unveiling		Water
Sample reveal		Water
Sample disclosure		Water
Sample exposure		Water
Sample introduction		Water
Sample presentation		Water
Sample display		Water
Sample exhibition		Water
Sample demonstration		Water
Sample presentation		Water
Sample unveiling		Water
Sample reveal		Water
Sample disclosure		Water
Sample exposure		Water
Sample introduction		Water
Sample presentation		Water
Sample display		Water
Sample exhibition		Water
Sample demonstration		Water
Sample presentation		Water
Sample unveiling		Water
Sample reveal		Water
Sample disclosure		Water
Sample exposure		Water
Sample introduction		Water
Sample presentation		Water
Sample display		Water
Sample exhibition		Water
Sample demonstration		Water
Sample presentation		Water
Sample unveiling		Water
Sample reveal		Water
Sample disclosure		Water
Sample exposure		Water

(Japan) 1989 10, Suppl. 3, 587-597, J. Cereb. Blood Flow Metab. 1990, 10, 221-6, Br. J. Radiol. 1990, 63, 120-4, Pediatr. Res. 1989, 25, 440-4, Radiology 1989, 170, 873-8, Cereb. Blood Flow Metab. 1988, 8, 816-821, J Neuro. Chem. 1988, U51U, 1501-9 abd Am. Heart J. 1988, 116 701-8. WO98/41241 of Nihon Schering discusses MRI techniques which utilise polymers in the monitoring of pH.

One important physiological parameter of great medical interest has been temperature; temperature has been measured by electron paramagnetic resonance spectroscopy (J. Biomech. Eng. 1996, 118, 193-200), an ytterbium chelate has been used as a temperature sensitive probe for MR spectroscopy (Magn. Res. Med. 1996, 35, 648-651), fast imaging techniques have been evaluated in MRI for temperature imaging (J. Magn. Reson. B, 1996, 112, 86-90), ^{31}P and ^1H magnetic resonance spectroscopy has been used to study relationship between brain temperature and energy utilization rate in vivo (Pediatr. Res. 1995, 38, 919-925), local brain temperature has been estimated in vivo by ^1H NMR spectroscopy (J. Neurochem. 1995, 38, 1995, 1224-30), magnetic resonance has been used to follow temperature changes during interstitial microwave heating (Med. Phys. 1997, 24, 269-277), the temperature dependence of canine brain tissue diffusion coefficient has been measured in vivo using magnetic resonance echoplanar imaging (Int. J. Hyperthermia 1995, 11, 73-86), temperature dependent ultrasound colour flow Doppler imaging has been carried out of experimental tumours in rabbits (Ultrasound Med. Biol. 1993, 19, 221-9), electrical impedance tomography has been proposed for temperature measurement (Trans ASME J. Biochem. Eng. 1996, 118, 193-200), temperature measurement has been carried out in vivo using a temperature-sensitive lanthanide complex and ^1H magnetic resonance spectroscopy (Magn. Res. Med. 1996, 35,

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The following, predominantly MRI based, techniques have also been reported in the measurement of temperature and temperature changes; Med. Phys 1997, 24(2), 269-277, Int. J. Hyperthermia 1995, 11(5), 409-424, Int. J. Hyperthermia 1992, 8(2), 253-262, Int. J. Hyperthermia (1994), 10(3), 389-394, Radiologe 1998, 38, 200-209, Med Phys 1997, 24(12), 1899-1906, JMRI 1998, 8, 128-135, JMRI, 1998, 8, 160-164, JMRI 1998, 8, 165-174, MRM 1995, 34, 359-367, MRM 1995, 33, 729-731, MRM 1995, 33, 74-81, Radiology 1998, 208, 789-794, JMRI 1996, 7, 226-229, JMRI 1997, 8 188-196, JMRI 1998, 8, 197-202, JMRI 1998, 8, 31-39, JMRI 1998, 8, 121-127, JMRI 1998, 8, 493-502, Int. J. Radiation Oncology Biol. Phys. 1998,

40(4), 815-822, Int. J. Hyperthermia 1998, 14(5), 479-493, Radiology 1995, 196, 725-733, Advances in Radiation Therapy 1998 Eds. Mittal, Purdy and Ang, Kluwer Academic Publishers, Chapter 10, pp. 213-245.

Several patents and patent applications which relate to physiological imaging have been published: use of macrocyclic metal complexes as temperature probes for the determination of body temperature using spectroscopic methods with reduced background signals (WO94/27977); new fluorine containing macrocyclic metal complexes from tetraazadodecane derivatives useful for measuring tissue temperature from NMR chemical shift values, and as contrast agents for X-ray or NMR diagnosis (WO94/27978); determining and imaging of temperature change in human body using diffusion coefficients obtained by NMR to determine absolute temperature for individual points of body and temperature differences (WO90/02321); thermographic imaging using a temperature dependent paramagnetic material in an ESR enhanced magnetic resonance imaging apparatus (WO90/02343); fluorosubstituted benzene derivatives useful as agents for in vivo NMR diagnosis, e.g. for measurement of tissue specific pH temperature, redox potentials, etc. (EP-A-368429); a magnetic resonance pulsed heat system for selectively heating a region of a subject that uses pulsed heat from focussed ultrasound equipment to destroy tumor tissue and MRI to provide fast scan images to monitor tissue and temperature with a diffusion sensitive pulse sequence (US-A-5247935); a magnetic resonance pulsed heat system for selectively heating tissue - surgery is performed using localised heating of tissue guided by and monitored by temperature sensitive magnetic resonance imaging and body tissue is heated using a magnetic resonance imaging system having a source and a probe containing a magnetic imaging coil and heating imaging rf source (US-A-5323778); apparatus for hyperthermia

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Viewed from a further aspect the invention provides a parenterally administrable contrast medium for imaging of a physiological parameter, said medium comprising a particulate material the particles whereof comprise a matrix or membrane material and at least one contrast generating species, said matrix or membrane material being responsive to said physiological parameter to cause the contrast efficacy of said contrast generating

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the parameter value with time, or it may simply be a calculated numerical value for the parameter or an indication that the parameter is below or above a particular threshold value. Desirably, however, the signal provides a quantitative or at least semi-quantitative value for the parameter, e.g. either in a region of interest or in a plurality of regions of interest in the body, for example providing a spatial and/or temporal map of the parameter within at least a portion of the body.

Data relating to a physiological parameter may not necessarily also contain information relating to the anatomy of the animal body and thus, a further aspect of the invention relates to the combination of traditional anatomical imaging with physiological imaging to obtain two images, one containing information about a physiological parameter and the other containing anatomical information. The two images may be combined to give one image with both anatomical and physiological information.

Thus, according to a further aspect is provided a method of imaging of an animate human or non-human animal body, which method comprises:

administering parenterally to said body at least one contrast generating species the contrast efficacy whereof is responsive to a change in value of a pre-selected physiological parameter;

generating image data of at least part of said body in which said species is present; and

generating therefrom a signal indicative of the value or variation of said parameter in said part of said body and also generating an anatomical image of the same part of the animal body.

The additional use of anatomical information may aid interpretation of the physiological data. An image generated in response to a physiological parameter, a 'physiological image', may be formed using any of the

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imaging methods and or contrast media described herein. This physiological image can be combined with a conventional image obtained with or without a contrast agent. Suitable contrast agents for use with traditional anatomical imaging are well known in the art for all types of imaging techniques, MRI, X-ray, ultrasound, light and nuclear imaging etc. and many suitable contrast agents for anatomical imaging are discussed herein.

The imaging technique used to obtain physiological data may be the same or different to the imaging technique used to obtain the anatomical image. In a preferred embodiment the imaging technique will be the same, MRI being particularly suitable.

Two separate contrast agents may be used, one for physiological imaging and one for traditional imaging. The two agents can be injected sequentially and the body scanned sequentially with respect to the appropriate imaging techniques and optionally the two images which are generated are then combined. In an alternative embodiment, a single multi-functional contrast agent may be used which is capable of providing both physiological and anatomical information. A multi-functional MRI contrast agent may be used, wherein one of its functions responds to a physiological parameter while a second function provides anatomical information. Although a single contrast agent is applied, the body may be scanned twice and the resulting two images combined.

In a further alternative embodiment a multi-functional contrast agent may be used wherein the components of the agent function as contrast agents for different imaging techniques. Thus, the contrast agent may contain microbubbles to provide contrast in ultrasound imaging and paramagnetic complexes for MRI, one of these components being responsive to a physiological parameter. Again, the images obtained by scanning according to the two imaging techniques may be

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combined.

By way of a further example, MRI with hyperpolarised substances will tend to provide good physiological information relating to e.g. pH, temperature or pressure but little or no anatomical information. Thus, the hyperpolarised MR image is advantageously combined with an anatomical image, e.g. by superimposing the images. The two images may be generated separately or at the same time.

The combination of physiological and anatomical imaging may be used to investigate all parts of the human or non-human animal body and any of the physiological parameters discussed herein, particularly pH and temperature. Where the physiological parameter is temperature, changes in the value of the parameter, i.e. temperature changes, may be caused by intrinsic or extrinsic means. Intrinsic means will include cancer, cardiovascular disease and inflammation while extrinsic means include hyperthermia (external heating) treatment. Thus, the physiological contrast agent may be a contrast agent for hyperthermia.

The imaging technique used in the method of the invention may be any technique capable of use in conjunction with contrast agents, e.g. X-ray (e.g. CT scanning), MRI, MRS, MR microscopy, ESR imaging, ESR spectroscopy, Mössbauer imaging, ultrasound, light imaging, nuclear imaging (e.g. scintigraphy, PET or SPECT), MSI, APT, etc. In magnetic resonance techniques, signal strength or chemical shift or both may typically be studied. Preferably, the technique used will be an X-ray, MRI, ultrasound, light imaging or nuclear imaging technique (e.g. scintigraphy), in particular an MRI or ultrasound technique. The particulate contrast agent used will accordingly be or contain a material capable of having a contrast or signal generating effect in the particular imaging modality selected, e.g. a gas or gas precursor, a

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paramagnetic, ferromagnetic, ferrimagnetic or superparamagnetic material or a precursor therefor, hyperpolarized nmr active (ie. non zero nuclear spin) nuclei (e.g noble gas or ^{13}C nuclei), a radionuclide, a chromophore, (which term is used to include fluorescent and phosphorescent materials as well as light absorbers) or a precursor therefor, an ionic species, etc.

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The physiological parameter studied using the method of the invention may be any physiochemical parameter capable of affecting the matrix or membrane material of the contrast agent, e.g. pressure, temperature, pH, oxygen tension, carbon dioxide tension, enzyme activity, metabolite concentration, tissue electrical activity, tissue diffusion, ion concentration, particularly Mg^{2+} , Ca^{2+} and Zn^{2+} , etc. Preferably however it will be selected from blood parameters, e.g. pressure, temperature and pH, in particular in the vasculature rather than the chambers of the heart. Where temperature is being measured, changes may be due to intrinsic factors such as disease or because of external factors, i.e. hyperthermia. It is not envisaged that the parameter be one which does not affect the membrane or matrix, for example flow rate or perfusion density.

A key part of the present invention is that the contrast agent particles should comprise a membrane or matrix material which is responsive to the physiological parameter under investigation so as to alter the contrast efficacy of the contrast agent. The manner in which the membrane or matrix responds will depend on the particular combination of imaging modality, physiological parameter and contrast generating material selected. Typically however the response might involve a change in membrane or matrix permeability to one or more species (e.g. water or gases), chemical or physical breakdown of the membrane or matrix material, generation of a contrast generating material, cleavage of

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Another embodiment of the present invention relates to pH sensitive particulate compositions for determination of pH in the body. By way of example the active contrast agent (or indicator or probe) may be a paramagnetic, magnetic or fluorinated compound detectable by MRI. The active contrast agent (or

indicator or probe) may be a gas or a gas generating substance for detection by ultrasound, it may be a radioactive substance for detection by scintigraphy, SPECT or PET, or it may be a fluorescent dye, a near infrared dye, a UV dye or another dye that can be detected in vivo in light imaging or light detection methods.

Yet another embodiment of the invention relates to particulate compositions as contrast agents or as in vivo indicators or probes for detection of oxygen concentration/tension in the tissue using modalities such as ultrasound, MRI, Overhauser MRI and ESR.

Another embodiment of the present invention relates to particulate compositions as contrast agents or as in vivo indicators or probes for detecting pressure, turbulence, viscosity, enzyme activity, ion concentrations, metabolite diffusion coefficients, elasticity and flexibility.

Another aspect of the present invention relates to particulate compositions as contrast agents or as in vivo indicators or probes in combination with a targeting ligand, wherein said targeting ligand targets cells or receptors selected from the group consisting of myocardial cells, endothelial cells, epithelial cells, tumor cells, brain cells, and the glycoprotein GPIIb/IIIa receptor, for detection of changes in physiological parameters and/or quantification/semiquantification of physiological parameters relevant for diagnosis of disease.

Further examples of targeting ligands which can be used are:

i) Antibodies, which can be used as vectors for a very wide range of targets, and which have advantageous properties such as very high specificity, high affinity (if desired), the possibility of modifying affinity according to need etc. Whether or not antibodies will be bioactive will depend on the specific vector/target

combination. Both conventional and genetically engineered antibodies may be employed, the latter permitting engineering of antibodies to particular needs, e.g. as regards affinity and specificity. The use of human antibodies may be preferred to avoid possible immune reactions against the vector molecule.

A further useful class of antibodies comprises so-called bispecific antibodies, i.e. antibodies having specificity for two different target molecules in one antibody molecule. Such antibodies may, for example, be useful in promoting formation of bubble clusters and may also be used for various therapeutic purposes, e.g. for carrying toxic moieties to the target. Various aspects of bispecific antibodies are described by McGuinness, B.T. et al. in *Nat. Biotechnol.* (1996) **14**, 1149-1154; by George, A.J. et al. in *J. Immunol.* (1994) **152**, 1802-1811; by Bonardi et al. in *Cancer Res.* (1993) **53**, 3015-3021; and by French, R.R. et al. in *Cancer Res.* (1991) **51**, 2353-2361.

ii) Cell adhesion molecules, their receptors, cytokines, growth factors, peptide hormones and pieces thereof. Such vectors/targeting ligands rely on normal biological protein-protein interactions with target molecule receptors, and so in many cases will generate a biological response on binding with the targets and thus be bioactive; this may be a relatively insignificant concern with vectors which target proteoglycans.

iii) Non-peptide agonists/antagonists or non-bioactive binders of receptors for cell adhesion molecules, cytokines, growth factors and peptide hormones. This category may include non-bioactive vectors which will be neither agonists nor antagonist but which may nonetheless exhibit valuable targeting ability.

iv) Oligonucleotides and modified oligonucleotides which bind DNA or RNA through Watson-Crick or other types of base-pairing. DNA is usually only present in

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extracellular space as a consequence of cell damage, so that such oligonucleotides, which will usually be non-bioactive, may be useful in, for example, targeting of necrotic regions, which are associated with many different pathological conditions. Oligonucleotides may also be designed to bind to specific DNA- or RNA-binding proteins, for example transcription factors which are very often highly overexpressed or activated in tumour cells or in activated immune or endothelial cells. Combinatorial libraries may be used to select oligonucleotides which bind specifically to possible target molecules (from proteins to caffeine) and which therefore may be employed as vectors for targeting.

v) DNA-binding drugs may behave similarly to oligonucleotides, but may exhibit biological activity and/or toxic effects if taken up by cells.

vi) Various small molecules, including bioactive compounds known to bind to biological receptors of various kinds. Such vectors or their targets may be used to generate non-bioactive compounds binding to the same targets.

vii) Targeting ligands may be selected from combinatorial libraries without necessarily knowing the exact molecular target, by functionally selecting (in vitro, ex vivo or in vivo) for molecules binding to the region/structure to be imaged.

ix) Proteins or peptides which bind to glucosamino-glycan side chains e.g. heparan sulphate, including glucosaminoglycan-binding portions of larger molecules, since binding to such glucosaminoglycans side chains does not result in a biological response. Proteoglycans are not found on red blood cells, thus eliminating undesirable adsorption to these cells.

The particulate contrast agent may thus be used for quantification/semi-quantification of a physiological parameter which is relevant for diagnosis of disease. The particulate contrast agent may be triggered into

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giving a measurable signal difference either by the target parameter itself (e.g. the local temperature, pH or pressure or by binding to the particular cell surface receptors of interest) or by a chemical or biological response of the target parameter (e.g. release of enzymes or local variation in pH or temperature due to cellular reactions). The particulate agent may thus respond to, identify and/or quantitatively or semi-quantitatively determine bacteria, viruses, antibodies, enzymes, drugs, toxins, etc.

Another aspect of the present invention relates to intravenous particulate compositions as contrast agents or as in vivo indicators or probes with long vascular half life (reduced liver uptake) for detection of changes in physiological parameters and/or quantification/semiquantification of physiological parameters relevant for diagnosis of disease.

The particulate contrast agent used according to the invention may be a solid material, a porous material, a liquid crystal material, a gel, a plastic material, a material having one or more walls or membranes or liquid particles, e.g. emulsion droplets or gas based particles, e.g. micro bubbles. The particles can also be thermodynamically stabilised, e.g. micro emulsion droplets or surfactant micelles. The chemical composition of the particulate material can be one simple chemical compound or a mixture of two or more chemical compounds. Generally it will comprise two or more different chemical entities, at least one of which is a matrix or membrane forming material and at least one other of which is a contrast generating species. The composition can consist of solid material(s) only or it may be a mixture of different solids/liquids/gases. The particulate will generally have a mean particle size (e.g. as determined by particle size analyzers such as laser light scattering apparatus or Coulter counters) in the range 0.001 to 20 μm , more preferably 0.01 to 10 μm ,

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especially 0.05 to 7 μm . Such particles are often described in the literature as particles, colloids, emulsions, droplets, microcrystals, nanocrystals, microparticles, nanoparticles, vesicles, liposomes, bubbles, microspheres, microbubbles, coated particles, microballons and the like.

The term "polymer" as used herein refers to any chemical compound with more than 10 repeating units. A polymer can be naturally occurring, synthetic, or semisynthetic. Semisynthetic polymers are polymers that are produced by synthetic modification of naturally occurring polymers. Compounds with 2 to 10 repeating units are herein generally referred to as "oligomers" and likewise may be natural, synthetic or semisynthetic.

The term "surface active compound" or "surfactant" is used herein to refer to any chemical compound having at least one hydrophilic functional group and at least one hydrophobic (lipophilic) group. In a multiphase system, surface active compounds will commonly accumulate at the interface.

The term "lipid" is used herein to refer to naturally-occurring compounds, synthetic compounds and semisynthetic compounds which are surface active compounds and have structures similar to fatty acids, waxes, mono-, di- or tri-glycerides, glycolipids, phospholipids, higher (C_{10} or greater) aliphatic alcohols, terpenes and steroids.

The term "gas" is used herein to refer to any compound or a mixture of compounds with sufficiently high vapor pressure to be at least partly in the gas phase at 37°C.

When the imaging modality used according to the invention is ultrasound, the contrast generating species in the contrast agent will preferably consist of one or more encapsulated gases and/or one or more encapsulated gas precursors. This contrast generating species is able to interact with the surroundings so that the

contrast agent gives information about one or more physiological parameters generally as a result of an interaction between the surroundings and the encapsulation material, if necessary followed by changes related to the gas/gas-precursor. However gaseous contrast generating species may be used in other imaging modalities, such as MRI and X-ray for example.

Typical examples of gas types that change contrast property as a result of the physiological parameters in the surrounding tissue include: gases that are generated from a precursor as a result for example of pH, temperature or pressure changes, e.g. as a result of a chemical reaction, as a result of the boiling point of the gas, or as a result of a change of solubility; gases that compete with blood gases for absorption or adsorption sites within the matrix or membrane material; gases that change properties (e.g. lose hyperpolarization or change other magnetic properties) upon contact with body fluids or components, including dissolved components, thereof; gas molecules sensitive to pH; gases that change properties/volume with temperature; gases that change volume as a result of surrounding gas (e.g. oxygen tension); etc.

Preferred gases include hydrogen, oxygen, nitrogen, noble gases (including hyperpolarized gases), carbon dioxide, fluorinated gases (e.g. sulphur hexafluoride, fluorohydrocarbons, perfluorocarbons and other fluorinated halogenated organic compounds in gas phase), and low molecular weight hydrocarbons. Preferred gases also include any pharmaceutically acceptable gas mixture like for example air and air/perfluorocarbon mixtures. Preferably, the perfluorocarbon gas is selected from perfluoromethane, perfluoroethane, perfluoropropanes and perfluorobutanes. Any physiologically acceptable gas precursor can be used. Among suitable gas precursors are compounds that form a gas as a result of a chemical reaction (for example compounds sensitive to pH, for

example carbonic acid, aminomalonic acid or other acceptable pH sensitive gas generating substances). Other suitable gas precursors are compounds that form a gas as a result of other physiological conditions like for example temperature, oxygen, enzymes or other physiological parameters/compounds relevant for body tissue (whether in the normal or diseased state) or which are activated to a gas forming state as a result of an interaction with an external stimulus (e.g. photo-activation, sono-activation etc.).

The encapsulation material can be any material such as for example lipids, phospholipids, surfactants, proteins, oligomers and polymers. Such materials may be chosen to dissolve, melt, collapse, weaken, increase porosity, or otherwise break down, change phase or change size (e.g. by aggregation due to change in surface charge, for example in response to local Ca^{2+} and/or Mg^{2+} concentration) in response to the physiological parameter, e.g. to allow release of the contrast generating species into the surrounding fluid, or to allow body fluid or components thereof to come into contact with the contrast generating species, or to raise contrast agent species local concentration above the detection limit, etc. In this way the contrast generating effect of the contrast generating species may be dispersed (e.g. into the extracellular fluid space), switched on or increased (e.g. by generation of a contrast generating species such as a gas or by increasing water contact (for a positive (T_1 effect) MR contrast agent such as a gadolinium chelate)), or switched off or decreased (e.g. by destruction of the compartmentalization required for a negative (T_2 effect) MR contrast agent such as a dysprosium chelate, or by quenching of a radical or depolarization of a hyperpolarized nucleus or dissolution of a blood soluble gas). Moreover a porous solid matrix, e.g. a zeolite, may be impregnated with the contrast generating species

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with the pore mouths then being closed off totally or partially using a material which breaks down, melts or dissolves when the relevant physiological parameter (e.g. pH, temperature, enzyme concentration) in the surrounding body fluid is above or below a pre-set value.

The particulate contrast agent used according to the invention may respond to physiological parameters in several different ways. In one aspect, the particulate contrast agent may respond to physiological parameters by accumulation in the area where a certain value for a particular parameter is fulfilled, compared to areas where it is not. In another aspect of the invention, the particulate contrast agent responds by accumulation in areas where the physiological parameter value is not fulfilled. In yet another aspect of the invention, the particulate contrast agent responds to a given parameter by disintegration, the disintegration being dissolution or chemical breakdown. Especially advantageous is a response to a physiological parameter by leakage or other transport means in/out of the particles. The opposite situation where the response to a physiological parameter is to prevent dissolution/leakage by attaining an increase in stability/reduction in membrane transport compared to particles in areas where a threshold value for a given parameter is not fulfilled, is also a preferred aspect of the present invention. This type of response is advantageous since a time course may lead to a reduction in contrast by elimination from the organ in areas where the threshold value for the parameter is not fulfilled, while the contrast remains in the area of interest.

When a particulate composition responds by disintegration or transport, changes in contrast effect may be achieved by exposing otherwise invisible/shielded contrast agents, altering the distribution of contrast agents or, when the contrast agent is the particle

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itself (as in ultrasound contrast agents), destroying the contrast giving property. Especially advantageous are particulate compositions where the contrast effect is gained by interaction with the environment. In this case, both transport of the contrast agent and transport of the actual environmental component may be utilized for detection of physiological parameters. An example is MRI contrast agents where an increased degree of water access/transport to the contrast agent leads to the measured contrast enhancement. In this case, response to a physiological parameter may be an increased rate of water transport in/out of the particulate.

The leakage or an increased transport rate of solutes in/out of a particulate may be accomplished in a variety of ways. All kinds of phase transitions may be utilized to induce leakage/transport. For instance, a solid particle/membrane may become leaky when it is melted, the process being sensitive to temperature. Phase transitions involving a gas phase may be used to respond to pressure as a physiological parameter. An especially useful aspect of the present invention is particles comprising liquid crystalline material as for example liposomes, niosomes or other vesicles. Liquid crystalline materials may undergo several different phase changes which may induce leakage and/or increase the transport rate of solutes or even breakdown of the particle. For example, the gel to liquid crystalline phase transition of phospholipids may increase the liposome permeability and increase the transport rate or induce leakage of solutes on heating and hence temperature sensitivity. The lamellar to reversed hexagonal phase transition will also induce leakage since the liposomes require lipids in lamellar, gel or other layered phase structure. The lamellar to reversed hexagonal phase transition may be induced by pH, electrolytes, and changes in the chemical environment

姓名	性别	年龄	籍贯	职业	文化程度	健康状况	婚姻状况	子女情况	其他情况
王德胜	男	45	山东	工人	高中	良好	已婚	2子1女	无
李秀英	女	38	河北	教师	大学	良好	已婚	1子1女	无
张国强	男	52	河南	农民	初中	一般	已婚	3子2女	有
刘小红	女	28	江苏	护士	大专	良好	未婚	无	无
陈为民	男	60	浙江	退休	小学	较差	已婚	4子3女	有
赵大伟	男	35	广东	工程师	硕士	良好	已婚	1子1女	无
孙丽娟	女	42	湖北	医生	本科	良好	已婚	2子1女	无
周建民	男	55	四川	干部	高中	一般	已婚	3子2女	有
吴小芳	女	30	湖南	会计	大专	良好	未婚	无	无
郑为民	男	48	安徽	工人	初中	一般	已婚	2子1女	有
冯大刚	男	58	江西	农民	小学	较差	已婚	4子3女	有
马秀兰	女	33	山西	教师	大学	良好	已婚	1子1女	无
徐国强	男	40	陕西	工人	高中	良好	已婚	2子1女	无
郭为民	男	50	甘肃	干部	初中	一般	已婚	3子2女	有
李小红	女	25	福建	护士	大专	良好	未婚	无	无
周建民	男	65	广西	退休	小学	较差	已婚	4子3女	有
吴小芳	女	35	云南	会计	大专	良好	已婚	2子1女	无
郑为民	男	45	贵州	工人	初中	一般	已婚	2子1女	有
冯大刚	男	55	海南	农民	小学	较差	已婚	4子3女	有
马秀兰	女	30	重庆	教师	大学	良好	已婚	1子1女	无
徐国强	男	40	四川	工人	高中	良好	已婚	2子1女	无
郭为民	男	50	湖南	干部	初中	一般	已婚	3子2女	有
李小红	女	25	湖北	护士	大专	良好	未婚	无	无
周建民	男	65	江西	退休	小学	较差	已婚	4子3女	有
吴小芳	女	35	安徽	会计	大专	良好	已婚	2子1女	无
郑为民	男	45	浙江	工人	初中	一般	已婚	2子1女	有
冯大刚	男	55	江苏	农民	小学	较差	已婚	4子3女	有
马秀兰	女	30	山东	教师	大学	良好	已婚	1子1女	无
徐国强	男	40	河北	工人	高中	良好	已婚	2子1女	无
郭为民	男	50	河南	干部	初中	一般	已婚	3子2女	有
李小红	女	25	广东	护士	大专	良好	未婚	无	无
周建民	男	65	广西	退休	小学	较差	已婚	4子3女	有
吴小芳	女	35	云南	会计	大专	良好	已婚	2子1女	无
郑为民	男	45	贵州	工人	初中	一般	已婚	2子1女	有
冯大刚	男	55	海南	农民	小学	较差	已婚	4子3女	有
马秀兰	女	30	重庆	教师	大学	良好	已婚	1子1女	无
徐国强	男	40	四川	工人	高中	良好	已婚	2子1女	无
郭为民	男	50	湖南	干部	初中	一般	已婚	3子2女	有
李小红	女	25	湖北	护士	大专	良好	未婚	无	无
周建民	男	65	江西	退休	小学	较差	已婚	4子3女	有
吴小芳	女	35	安徽	会计	大专	良好	已婚	2子1女	无
郑为民	男	45	浙江	工人	初中	一般	已婚	2子1女	有
冯大刚	男	55	江苏	农民	小学	较差	已婚	4子3女	有
马秀兰	女	30	山东	教师	大学	良好	已婚	1子1女	无
徐国强	男	40	河北	工人	高中	良好	已婚	2子1女	无
郭为民	男	50	河南	干部	初中	一般	已婚	3子2女	有
李小红	女	25	广东	护士	大专	良好	未婚	无	无
周建民	男	65	广西	退休	小学	较差	已婚	4子3女	有
吴小芳	女	35	云南	会计	大专	良好	已婚</		

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such as targeting, enzymes, antibodies etc. The suitable parameter to respond to may be tuned by selection of the membrane composition and processing. Other phase transitions such as lamellar to cubic phases, lamellar to microemulsion phases or lamellar to normal hexagonal phase may also be used to introduce leakage.

Gel based particles or gel-surrounding particles (e.g. particles made by coacervation) may respond to a physiological parameter by, for example, a lowering of the viscosity of the gel. Such viscosity lowering may for example be obtained by temperature, pH or electrolytes such as Ca^{2+} or Mg^{2+} and the particles are thus sensitive to these parameters. Such parameters may also induce phase separation in the gel particles, leading to leakage of liquid and phase separation of the polymer which comprises the gel. These mechanisms may in turn influence a parameter such as water leakage and exposure of, e.g. paramagnetic chelates to water and hence lead to a change in MRI contrast.

Particles or membranes composed of solid polymer may also respond to physiological parameters. For instance temperature may change the glass transition temperature of the polymer, and hence induce phase transitions in the polymer membrane, which in turn may influence a parameter such as water transport which influences the contrast efficacy of the contrast agent.

Particles which at least in part are composed of or stabilised by water soluble polymers e.g. peptides, may respond to physiological parameters by alternation in the peptide conformation. For instance peptides may undergo an α - helix to β - sheet transition or vice versa and hence influence a parameter which in turn effects contrast. Also transitions to/from α - helix or β - sheet to random coil may influence a parameter such as membrane permeability, particle stability against aggregation/flocculation or even fusion, or particle

dissolution or precipitation which in turn alters the contrast efficacy of the contrast agent.

Also of use as contrast agents when the imaging modality is ultrasounds are temperature and pressure sensitive emulsions and fluids.

Leakage may also be controlled by entities forming channels or other transport routes through the membrane of a particle. These channels may control the transport of molecules in/out of the particle, and be quite selective for, e.g., ions. For instance the protein tubulin which forms microtubules in absence of Ca^{2+} may induce a higher leakage in presence of Ca^{2+} than in absence of Ca^{2+} and hence be Ca^{2+} sensitive. Other proteins/enzymes which may control the transport of substance in/out of a vesicle, include erythrocyte anion transporter, erythrocyte glucose transporter, Na^+/K^+ ATPase (Na^+/K^+ pump), Ca^{2+} - ATPase (Ca^{2+} pump) and Bacteriorhodopsin (H^+ - pump). Also biosurfactants such as iturins, esperine, bacillomycins, mycosubtilin, surfactin and similar substances may be used as membrane components to induce/prevent leakage by response to external parameters since these molecules may respond by changes in secondary and tertiary structure as well as self-assembly properties on influence from extrinsic parameters.

The contrast generating species in MR contrast agents used according to the invention will generally be a paramagnetic, superparamagnetic, ferrimagnetic or ferromagnetic compound and/or a compound containing other non zero spin nuclei than hydrogen, e.g. ^{19}F , ^{13}C , ^{15}N , ^{29}Si , ^{31}P and certain noble gases, such as ^{129}Xe or ^3He .

Preferred as paramagnetic compounds are stable free radicals, and compounds (especially chelates) of transition metal or lanthanide metals, e.g. manganese compounds, gadolinium chelates, ytterbium chelates and dysprosium chelates. Preferred magnetic (e.g.

Examples of MR contrast effective materials are well known from the patent literature, see for example the patent publications of Nycomed, Salutar, Sterling Winthrop, Schering, Squibb, Mallinckrodt, Guerbet and Bracco.

Typical examples of species that change contrast property as a result of the physiological parameters in the surrounding tissue include: paramagnetic chelates that change relaxation properties and/or change chemical shift as a result of temperature, paramagnetic chelates that change coordination number and thereby relaxation properties and/or shift properties as a function of pH, paramagnetic compounds, for example manganese compounds ($Mn(2+)/Mn(3+)$), europium compounds ($Eu(2+)$, $Eu(3+)$) and free radicals (radical, no radical) that change relaxation properties and/or shift properties as a result of oxygen tension/concentration or as a result of redox potential in the surrounding tissue, paramagnetic

and magnetic compounds that change relaxation/shift properties as a result of enzymic activity (for example with enzymatic cleavage of paramagnetic chelates from macromolecules conjugated thereto causing a change in correlation time and/or water coordination) and paramagnetic chelates that change properties as a result of concentration of ions in the tissue, e.g. due to changes in water coordination.

Paramagnetic compounds have, according to the present invention, either an effect on the relaxation times (T_1 or T_2) or an effect on chemical shift. Typical compounds that change relaxation times are gadolinium chelates, manganese compounds and superparamagnetic iron oxides. Europium chelates, on the other hand, are well-known chemical shift compounds. The effect on chemical shift is related to temperature. Based on this, macrocyclic paramagnetic chelates like 2-methoxyethyl substituted PrDO3A and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) thulium complex have been suggested as temperature probes (see WO 94/27977 (Platzek, Schering) and C.S. Zuo et al. in J. Magn. Res. **133** 53-60 (1998)). All these paramagnetic compounds can be used according to the present invention.

The contrast generating species in X-ray contrast agents for use according to the invention will generally be a gas or gas generator or a water-soluble compound containing heavy atoms (e.g. atomic number of 37 or greater), e.g. metal chelates, metal clusters, metal cluster chelates and iodinated compounds. Preferred contrast generating species include ionic and non-ionic iodinated organic aromatic compounds, in particular triiodophenyl compounds. Most preferred are approved iodine based contrast agents such as salts, e.g. sodium or meglumine salts, of iodamide, iothalamate, diatrizoate, ioxaglate and metrizoate, and non-ionics such as metrizamide (see DE-A-2031724), iopamidol (see

Such contrast generating species may be incorporated into matrices or coatings that are sensitive to one or more physiological parameter.

Such contrast generating species may be incorporated into matrices or coatings that are sensitive to one or more physiological parameter.

In vivo temperature measurements have been of great interest because temperature is an important physiological parameter related to several indications including cancer, cardiovascular diseases and

inflammation. Local monitoring of temperature will also be of great value during hyperthermia treatment.

Contrast generating species can be released from the matrix/encapsulation material as a result of increased temperature and thereby change their contrast property or distribute to other tissues than the particulate product. Alternatively for an MR active temperature sensitive agent, a change in contrast efficacy may occur due to an increased permeability of the matrix/encapsulation material, and, hence, to an increased rate of water transport across the matrix/encapsulation material, even if the agent itself does not leave the matrix/encapsulation material.

Typical examples of temperature sensitive particulate materials are temperature sensitive liposomes, these being especially suitable for use with MRI. These liposomes take advantage of the fact that the membrane permeability is markedly increased at the gel-to-liquid crystal phase transition temperature (T_c) of their membrane lipids. Also, possibly depending upon the membrane properties and the nature of the MR active agent, leakage of the agent may occur. Liposomes made from specific phospholipids or a specific blend of phospholipids may be stable up to 37°C but exhibit an increased water permeability or/and leak as they pass through an area of the body in which the temperature is raised, e.g. to 40 to 45°C, as a result of a disease process or an external heating. Table 1 below shows the transition temperature of various saturated phosphatidylcholines.

Table 1

Phosphatidylcholines (PC)	Transition temperature T_c (°C)
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12:0	-1
13:0	14
14:0	23
15:0	33
16:0	41
17:0	48
18:0	55
19:0	60
20:0	66
21:0	72
22:0	75
23:0	79
24:0	80

Table 2 below shows the phase transition of various unsaturated phosphatidylcholines.

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Variable	Mean	SD	Min	Max	Median	Mode	Skewness	Kurtosis	Shapiro-Wilk	Normality
Age	35.2	12.5	18	65	32	30	0.15	2.8	0.98	Normal
Gender	1.2	0.4	1	2	1	1	0.05	1.2	0.99	Normal
Marital Status	2.1	0.8	1	3	2	2	0.10	2.5	0.97	Normal
Education	15.8	2.1	10	20	16	16	0.08	2.9	0.98	Normal
Income	12500	8500	5000	30000	10000	10000	0.20	3.5	0.95	Normal
Occupation	1.5	0.5	1	3	1	1	0.05	1.2	0.99	Normal
Health Status	2.5	0.6	1	3	2	2	0.10	2.5	0.97	Normal
Stress Level	3.2	1.1	1	5	3	3	0.15	2.8	0.98	Normal
Life Satisfaction	4.1	0.9	3	5	4	4	0.05	1.2	0.99	Normal
Work-Life Balance	3.8	1.0	2	5	4	4	0.10	2.5	0.97	Normal
Family Support	4.5	0.8	3	5	4	4	0.05	1.2	0.99	Normal
Community Involvement	2.8	0.7	1	4	3	3	0.10	2.5	0.97	Normal
Personal Growth	3.5	0.9	2	5	3	3	0.15	2.8	0.98	Normal
Relationship Quality	4.2	0.8	3	5	4	4	0.05	1.2	0.99	Normal
Overall Well-being	3.9	1.0	2	5	4	4	0.10	2.5	0.97	Normal

Table 4

Phosphatidylglycerols (PG)	Transition temperature T _c (°C)
12:0	-3
14:0	23
16:0	41
18:0	55

Tables 1-4 are based on information from the product catalogue of Avanti Polar Lipid Inc., USA.

Accordingly, phospholipids or blends of phospholipids may be selected to give products with the correct T_c for thermosensitive liposomes for diagnostic use. Typical blends for preparation of thermosensitive liposomes for diagnostic use are mixtures of dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidyl glycerol (DPPG) and distearylphosphatidylcholine (DSPC).

Particulate contrast agents may also respond to temperature by utilizing the conformational temperature sensitivity of certain polymer systems. An example is poly(N-isopropyl acrylamide) which phase separates at 37°C. Hence particles comprising contrast agents will become leaky dependent on temperature (see Hoffmann et al. Macromol. Symp. 118: 553-563 (1997)).

Other examples of temperature sensitive matrices/coatings are lipid suspensions/emulsions containing the contrast generating species or other particulate or particulate like formulations that release the contrast generating species or change properties as a result of changes in temperature.

If the parameter under study is capable of manipulation, e.g. by treatment with drugs, external application of heat etc., it may be used to study the efficacy of such treatment or localized such treatment may be used to cause a change in contrast efficacy which

[illegible]

$$\frac{\delta T}{\delta t} r_l C_l + w_b c_b (T - T_a) = k \nabla^2 T + (Q_p + Q_m)$$

The temperature response of thermosensitive MR-liposomes can in general be divided into three distinct regions:

- c) 'high relaxivity' region; $r_1 = r_1^{\text{high}}$; $T > T_b$;
where r_1^{high} is a constant with T (ideally, $r_1^{\text{high}} \gg r_1^{\text{low}}$).

It is possible to quantify the local temperature in the temperature active region of the liposomes, provided

three criteria are met:

1. A well defined relationship exists around T_c between liposomal relaxivity and temperature; i.e. $r_1(T) = f(T)$; $T_1 < T < T_2$; where $T_1; T_2$ is a clinically relevant temperature range. Ideally r_1 should be a linear function of T over the range $T_1; T_2$.
2. The temperature active region covers a large enough temperature range.
3. The Gd concentration in tissue $[Gd]$ is known.

If $[Gd]$ is not known, even a qualitative assessment of temperature changes may prove difficult, since regions with different $[Gd]$ would have a different degree of enhancement, even if the temperature were the same. Furthermore, it would be impossible to say whether lack of enhancement after heating was due to a low local temperature or the absence of the liposomes in that region.

However the local $[Gd]$ *in vivo* can be estimated, based on the relaxation effects of the liposomes in the 'low relaxivity' state, by the following method:

1. Acquire quantitative R_1 and/or R_2/R_2^* images ($R_{1,2} = 1/T_{1,2}$) before contrast administration and after contrast administration but before hyperthermia is initiated. R_1 and R_2/R_2^* images can be routinely acquired on most state-of-the-art clinical MR systems.
2. Measure the fractional change in R_1 and/or R_2/R_2^* , $\Delta R_1 = R_1^{\text{post}} - R_1^{\text{pre}}$ in the region of interest.
3. The local Gd concentration is then given by: $[Gd] = \Delta R_1 / r_1$.
4. If r_1 is not known, the ratio of the Gd concentration between two regions is given by: $[Gd_1] / [Gd_2] = \Delta R_1 / \Delta R_2$. Alternatively, R_2 or R_2^* images can be used to obtain the same Gd ratio.

In general therefore, the absolute $[Gd]$ can not be determined, unless the liposomal relaxivity in the tissue is known. However this may be fairly well

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approximated for the r relaxivity, but not the r^* relaxivity, since this depends on tissue geometry. Nonetheless, the $[Gd]$ in one region relative to another can be estimated as described above. The relative $[Gd]$ is valuable information that can be used to adjust the signal enhancement in the image so that it reflects actual temperature changes. In order for this to be possible, one needs to assume that a 'core region' exists where the temperature is above T_c . It is likely in a clinical situation that such a core region exists where the heating is most efficient surrounded by a 'penumbra' where heating is less efficient and the temperature distribution is less well defined. Now, by knowing the relative $[Gd]$ in the core versus the penumbra, the image intensity can be adjusted to compensate for any difference in $[Gd]$ in the two regions.

It is possible to estimate $[Gd_1]/[Gd_2]$ using a strongly T_1 -weighted sequence in which case change in signal intensity is almost linearly related to change in R_1 and hence $[Gd]$. This requires $TR \ll T_1$ of the target tissue. Similarly, strongly T_2 or T_2^* -weighted sequences can be used to estimate $[Gd_1]/[Gd_2]$.

Thus, when a Gd compound is encapsulated in liposomes, the resulting relaxivity (r_1, r_2) is small due to restricted water access to the paramagnetic centre. However, given that a very T_1 -sensitive sequence is used, it is still possible to detect a change in T_1 due to the presence of the liposomes prior to heating (i.e. at temperatures well below T_c). Consequently, by acquiring quantitative R_1 - or T_1 -maps of the area of interest before and after contrast injection (prior to hyperthermia treatment), the change in R_1 or T_1 enables the local Gd concentration $[Gd]$ to be determined. After heating, regional variations in $[Gd]$ can thus be accounted for; variations in contrast enhancement due to temperature differences can therefore be distinguished from variations in contrast enhancement due to

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concentration variations.

The longitudinal relaxation rate R_1 after contrast administration is given by:

$$R_1 = R_1 + [Gd] \cdot r_1;$$

Where R_1 is the relaxation rate prior to contrast administration. The change in R_1 due to the contrast agent is therefore:

$$\Delta R_1 = R_1 - R_1 = [Gd] \cdot r_1;$$

After hyperthermia, a new R_1 - or T_1 -map is generated.

In conclusion, therefore, given that the T_1 effect of the liposomes is detectable below T_c , it is possible to map the local Gd concentration and consequently compensate for differences in contrast enhancement after heating due to local variations in Gd concentration. The R_2 or R_2' effect of the liposomes can also be used for this purpose.

In vivo pH measurements have been of great interest because pH is an important physiological parameter associated to several severe diseases. The pH value is usually reduced during cancer diseases, cardiovascular diseases like for example stroke, osteoporosis, inflammations and autoimmune diseases.

One type of pH sensitive encapsulation for diagnostic agents involves the use of pH sensitive liposomes. The general strategy is to employ pH-sensitive groups in the liposomal membrane. Such typical groups have pKa values between 4 and 5.5. Phospholipids useful for preparation of pH-sensitive diagnostic agents include diheptadecanoyl phosphatidylcholine (DHPC) in admixture with DPPC and N-palmitoyl homocystein (PHC) in different ratios (see Eur. J. Pharm. Biopharm. 1993, 39, 97-101 for a general review on temperature and pH-sensitive liposomes).

Another type of pH-sensitive encapsulation of contrast generating species involves the use of pH-sensitive surfactants like for example N-dodecyl-2-

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imidazole propionate (DIP) which has pKa of 6.8 (see for example Pharm. Res. 1993, 13, 404). This means that DIP at pH 7.3-7.4 (physiological) is in the non-ionized (non/low surfactant activity) form (80%) while at for example lysosomal pH (5.2) over 97% will be in the charged form.

Another means of pH-sensitive encapsulation of contrast generating species involves the use of matrix materials and/or coating materials with pKa values in the range of 4.5-7.0 so that the material is soluble or partly soluble in the charged form and insoluble or partly insoluble in the non-charged form. Such compounds can be physiologically acceptable low molecular weight compounds or physiologically acceptable polymers.

Still another means of pH-sensitive encapsulation involves the use of compounds that are chemically cleaved as a result of pH, for example polyorthoesters or polyacetals/ketals which are cleaved under acidic conditions.

Liposomes comprising phosphatidyl ethanolamines (PE) as the central component are another example of liposomes which can undergo a phase transition and become leaky when pH is reduced. pH sensitive liposomes can also be achieved by incorporation of fatty acids into phospholipid membranes.

In principle any charged particulate system where the charge is pH dependent and influences the packing of the membrane material can be used.

Access to oxygen is critical for all types of cells, and diagnostic agents for determination of oxygen concentration/tension in tissue will be of great importance in diagnosis of diseases like cancer, cardiovascular diseases, autoimmune diseases and several diseases in the central nervous system.

One type of oxygen or redox sensitive encapsulation/coating material is a material that has

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Determination of concentration of physiologically important ions in tissue is important for several diseases.

An example of $\text{Ca}^{2+}/\text{Mg}^{2+}$ sensitive particulate compositions are liposomes enriched with the dimeric phospholipid cardiolipin. A cardiolipin containing membrane may undergo a lamellar to reversed hexagonal phase transition upon addition of the divalent cations since these ions bind to the cardiolipin di-phosphatidyl group.

Some particles or stabilising membranes surrounding particles may also respond with a phase transition when exposed to Ca^{2+} or Mg^{++} . An example are liquid crystalline based particles e.g. liposomes, which may respond by a lamellar to reversed hexagonal phase

transition upon addition of Ca or Mg. Also gel particles may respond easily to Ca or Mg by a significant lowering of viscosity or even phase separation of the polymer which forms basis for the gel on exposure to Ca or Mg. This viscosity reduction or phase separation may induce a change in contrast effect.

Types of enzyme sensitive encapsulation material include matrices or coatings that are degraded by enzymes, for example simple esters of low molecular weight compounds or polyesters like polyacetic acid and others.

Various metabolites may also change the properties of coating materials.

Particulates can be made sensitive to for example antibodies based on enhanced leakage due to a phase transition induced by the chemical binding between membrane molecules and the antibody. As an example, liposomes comprising N-(dinitrophenylamino-e-caproyl)-phosphatidyl ethanolamine (DNP-cap-PE) become leaky due to a lamellar to reversed hexagonal phase transition when binding to anti-DNP. Another example includes liposomes comprising human glycophorin A in dioleoyl phosphatidyl ethanolamine membranes. These liposomes become leaky when immobilized antibodies are added.

A further aspect of the present invention is to use one of the above described particulate diagnostic agents together with another compound that has the potential to change the physiological parameter of interest or together with use of an external energy source to change the parameter of interest.

Thus one example is to administer thermosensitive diagnostic agents in connection with an external heating and to follow the heating effect in parts of the body.

Another example is to administer compounds that change pH in connection with a pH-sensitive particulate diagnostic agent to follow the pH-profile in the area of interest.

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Still another example is to cause the subject under study to inhale oxygen, after administration of an oxygen sensitive diagnostic agent, to follow oxygen uptake in tissue.

Early diagnosis is very important to obtain good therapeutic results. In most disease processes changes in physiological parameters take place before changes in morphology. All existing contrast agents diagnose morphology. The new types of contrast agent according to the invention are able to detect diseases at a very early stage in the disease process and thereby improve the therapeutic outcome for the patient.

Where the particulate diagnostic agent or a component thereof carries an overall charge, it will conveniently be used in the form of a salt with a physiologically acceptable counterion, for example an ammonium, substituted ammonium, alkali metal or alkaline earth metal cation or an anion deriving from an inorganic or organic acid. In this regard, meglumine salts are particularly preferred.

The diagnostic agents of the present invention may be formulated in conventional pharmaceutical or veterinary parenteral administration forms, e.g. suspensions, dispersions, etc., for example in an aqueous vehicle such as water for injections.

Such compositions may further contain pharmaceutically acceptable diluents and excipients and formulation aids, for example stabilizers, antioxidants, osmolality adjusting agents, buffers, pH adjusting agents, etc.

Where the agent is formulated in a ready-to-use form for parenteral administration, the carrier medium is preferably isotonic or somewhat hypertonic.

Where the particulate agent comprises a chelate or salt of an otherwise toxic metal species, e.g. a heavy metal ion, it may be desirable to include within the formulation a slight excess of a chelating agent, e.g.

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as discussed by Schering in DE-A-3640708, or more preferably a slight excess of the calcium salt of such a chelating agent.

The dosage of the diagnostic agents of the invention will depend upon the imaging modality, the contrast generating species and the means by which contrast enhancement occurs (e.g. with switching on or off of contrast, with dispersion of contrast out of the vascular space, etc).

In general however dosages will be between 1/10 and 10 times the dosage conventionally used for the selected contrast generating species or analogous species in the same imaging modality. Even lower doses may also be used.

While the present invention is particularly suitable for methods involving parenteral administration of the particulate material, e.g. into the vasculature or directly into an organ or muscle tissue, intravenous administration being especially preferred, it is also applicable where administration is not via a parenteral route, e.g. where administration is transdermal, nasal, sub-lingual or is into an externally voiding body cavity, e.g. the gi tract, the bladder, the uterus or the vagina. The present invention is deemed to extend to cover such administration.

The disclosures of all the documents mentioned herein are incorporated by reference.

The present invention will now be illustrated further by reference to the following non-limiting Examples.

Example 1

Preparation of Temperature Sensitive Paramagnetic Liposomes

Liposomes containing GdHPDO3A (ProHance, Bracco Spa, Milan, Italy) and GdDTPA-BMA (Omniscan, Nycomed Amersham Imaging AS, Oslo, Norway) were prepared by the

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thin film hydration method. Two different saturated phospholipid blends were used; one consisting of hydrogenated phosphatidyl choline (HPC) (Lipoid GmbH, Ludwigshafen, German) and hydrogenated phosphatidylserine-sodium (HPS) (NOF Corporation, Amagasaki, Japan); the other composed of DPPC and DPPG-sodium (Sygena Ltd, Liestal, Switzerland). The phospholipid mixtures contained 5% or 10% (w/w) of the negatively charged HPS and DPPG components. The phospholipid mixtures were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. DPPC/DPPG liposomes were formed by hydrating the lipid film with a pre-heated (55°C) aqueous solution (pH 7.4) of 250 mM GdDTPA-BMA or 250 mM Gd HPD03A. The HPC/HPS liposomes were prepared analogously but with a lipid hydration temperature of 70°C. The DPPC/DPPG and HPC/HPS liposomes were allowed to swell for 2 hours at 55 and 70°C respectively. The total lipid concentration was 50 mg/ml. The liposomes were subjected to 3 freeze-thaw cycles in liquid nitrogen. Differently sized liposomes were produced by sequential extrusion (Lipex Extruder[®], Lipex Biomembranes Inc., Vancouver, Canada) through polycarbonate filters of various pore diameters. The extrusion temperature was 55 and 70°C for the DPPC/DPPG and HPC/HPS liposomes respectively. Untrapped metal chelate was removed by gel filtration or dialysis against isoosmotic and isoprotic glucose solution.

Physiochemical Properties

The mean hydrodynamic diameter of the liposomes varied from 103 nm to 276 nm, as measured by photon correlation spectroscopy (ZetaSizer IV, Malvern Instruments Ltd., Malvern, England); the zeta potential was negative in the order of -25 mV, as determined by laser Doppler velocimetry at 25°C (ZetaSizer IV, Malvern Instruments Ltd., Malvern, England). The mean

gel-to-liquid crystalline phase transition temperature (T_c) of the HPC/HPS and DPPC/DPPG preparations was 50 and 42°C, respectively, as determined by differential scanning calorimetry (DSC4, Perkin Elmer Inc., Norwalk, CT).

Temperature Response of *In Vitro* MR Contrast Efficacy

Figure 1 of the accompanying drawings and Table 5 below show the temperature sensitivity of *in vitro* T_1 relaxivity (r_1) for liposome encapsulated GdDTPA-BMA and GdHPDO3A, respectively (0.47T). Figure 2 of the accompanying drawings shows the temperature response of the *in vitro* MR signal intensity for liposome encapsulated GdDTPA-BMA.

Figure 3 of the accompanying drawings shows a series of T_1 -w GRE images prior to and after heating of a gel phantom containing inserts of liposome encapsulated GdDTPA-BMA.

Table 5

Temperature (°C)	r_1 ($s^{-1} \text{ mM}^{-1}$)		
	DPPC/DPPG	HPC/HPS	Control
	103 nm	130 nm	
20	0.16	0.06	4.53
25	0.23	0.08	4.27
30	0.31	0.12	3.94
37	0.69	0.21	3.75
45	3.30	0.53	3.07
55	3.10	3.00	2.82
60	-	2.96	2.54

non-liposomal GdHPDO3A

Example 2

GdDTPA-BMA encapsulated within DSPC/DPPC/DPPG liposomes
 DSPC/DPPC/DPPG (weight ratio; 28.5/66.5/5)

liposomes were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. Liposomes were formed by hydrating the lipid film with a pre-heated (57°C) aqueous solution (pH \approx 7) of 250 mM GdDTPA-BMA (10 ml). The liposomes were subjected to 3 freeze-thaw cycles and allowed to swell for one and a half hours at 65°C. The liposome dispersion was extruded at 65°C through polycarbonate filters of various pore diameters. The liposome size (z-average) after extrusion was 167 nm. Untrapped GdDTPA-BMA was removed by dialysis against isoosmotic and isoprotic glucose solution.

Table 6 shows the temperature sensitivity of the *in vitro* r_1 (0.235T) in glucose 5% solution for liposome encapsulated GdDTPA-BMA.

Table 6

Temperature (°C)	r_1 in glucose 5% (s ⁻¹ mM ⁻¹)
30	0.098
35	0.13
38	0.22
40	0.27
41	0.31
43	1.10
45	2.92

Example 3

GdDTPA-BMA encapsulated within DPPC/DPPG/DPPE-PEG-2000 liposomes

DPPC/DPPG/DPPE-PEG-2000 (weight ratio; 90/5/5) liposomes were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was

[illegible]

Table 7

Temperature ($^{\circ}\text{C}$)	r_1 in glucose 5% ($\text{s}^{-1}\text{mM}^{-1}$)
35	0.32
37	0.46
38	0.56
39.2	2.53
40	4.16
42	5.65

GdDTPA-BMA encapsulated within DSPC/DPPC/DPPG liposomes

DSPC/DPPC/DPPG (weight ratio; 43/52/5) liposomes were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. Liposomes were formed by hydrating the lipid film with a pre-heated (63°C) aqueous solution (pH \approx 7) of 250 mM GdDTPA-BMA (10 ml). The liposomes were subjected to 3 freeze-thaw cycles and allowed to swell for one and a half

hours at 64 C. The liposome dispersion was extruded at 65 C through polycarbonate filters of various pore diameters. The liposome size (z-average) was 145 nm. Untrapped metal chelate was removed by dialysis against isoosmotic and isoprotic glucose solution.

Table 8 shows the temperature sensitivity of the *in vitro* r_i (0.235T) in both glucose 5% solution and human serum for liposome encapsulated GdDTPA-BMA.

Table 8

Temperature (°C)	r_i in glucose 5% (s ⁻¹ ·mM ⁻¹)	r_i in serum (s ⁻¹ ·mM ⁻¹)
35	0.12	0.14
40	0.22	0.25
42	0.29	0.44
44	0.88	1.91
46	4.47	4.51
48	4.40	4.51
50	4.40	4.35

Example 5

GdDTPA-BMA encapsulated within DPPC/DPPG liposomes

DPPC/DPPG (weight ratio; 95/5) liposomes were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. Liposomes were formed by hydrating the lipid film with a pre-heated (52°C) aqueous solution (pH \approx 7) of 250 mM GdDTPA-BMA (10 ml). The liposomes were subjected to 3 freeze-thaw cycles and allowed to swell for one and a half hours at 55°C. The liposome dispersion was extruded at 62°C through polycarbonate filters of various pore diameters. The liposome size (z-average) after extrusion was 148 nm. Untrapped metal chelate was removed by dialysis against isoosmotic and isoprotic glucose

solution.

Table 9 shows the temperature sensitivity of the *in vitro* r_1 (0.235 T) in both glucose 5% solution and human serum for liposome encapsulated GdDTPA-BMA.

Table 9

Temperature (°C)	r_1 in glucose 5% (s ⁻¹ mM ⁻¹)	r_1 in serum (s ⁻¹ mM ⁻¹)
35	0.331	0.389
38	0.753	0.810
39	1.47	1.20
40	3.75	3.31
41	4.88	5.05
42	4.80	4.99
44	4.80	4.78
48	4.77	4.88

Example 6

"Double transition" with a mixture of DSPC/DPPC/DPPG and DPPC/DPPG liposomes, containing both GdDTPA-BMA

1.5 ml liposomes from Example 4 were mixed with 1.5 ml DPPC/DPPG liposomes prepared as Example 5. The mixture was diluted to 40 ml with glucose 5% solution.

Table 10 shows the temperature sensitivity of the *in vitro* R_1 (0.235 T) in glucose 5% solution for the liposome mixture.

Table 10

Temperature (°C)	R. in glucose 5% (s ⁻¹)
35	2.46
38	2.61
39	2.83
40	3.87
41	7.11
42	7.17
44	10.9
46	14.0
48	14.0

Example 7Perfluorobutane bubbles stabilised by 5mg/ml
DSPC/DPPC/DPPG

DSPC/DPPC/DPPG (weight ratio; 28.5/66.5/5), perfluorobutane gas bubbles were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. The lipid film was hydrated for 1 hour at 60°C after addition of 100 ml 1.5 % propylene glycol in water. The final dispersion contained 5 mg lipids/ml.

Five 2 ml vials were filled with 1 ml of the dispersion. The headspace was flushed with perfluorobutane gas. The vials were shaken on a CapMixer for 45 seconds and left on a roller table over night. The content of the five vials were collected and centrifuged for 5 minutes at 2000 rpm. The infranatant was removed and replaced by the same volume water. The microbubbles were reconstituted by gentle handshaking after flushing the headspace with perfluorobutane gas. The washing procedure was repeated three times.

The sample of perfluorobutane bubbles was characterized using Coulter Multisizer II fitted with an aperture of 50 μ m and Nycomed in-house equipment for measuring acoustic attenuation. The dispersion showed a size distribution of volume median diameter of about 3 μ m. The bubbles showed a nice attenuation spectrum in the range 3.5-8.0 MHz and were tested for pressure stability at an over-pressure of 150 mm Hg in the temperature range 22-47°C using the acoustic technique. The acoustic measurements showed that the gas bubbles disrupted at an over-pressure of 150 mm Hg at 47°C, whereas they remained stable at 40°C. This indicates that the gas microbubbles can be used in ultrasound imaging for *in vivo* mapping of physiological pressure.

Example 8Imaging studies in rats with GdDTPA-BMA encapsulated within DPPC/DPPG liposomes**a) Intramuscular injection in the left thigh**

Liposomes were injected intramuscularly at a dosage of 0.02 mmol/kg. The left thigh muscle was heated with focused ultrasound whereas the right thigh muscle served as a control.

Figures 4-5 show axial T₂-w SE images of the thigh before and after liposome injection, respectively. Figures 6-8 are T₂-w SE images after 2, 5, and 9 minutes of heating, respectively.

At that timepoint, heating was terminated, the rat was removed from the MRI scanner and the temperature of the muscle was measured to be 47°C. Figure 9 represents the final image 15 minutes after termination of heating. For comparative purposes, the syringe containing the liposomal dispersion (identical to that injected) was included.

The results indicate that the signal intensity of the left thigh muscle increases substantially after heating, as compared to the right thigh muscle and syringe.

b) Intravenous injection

Liposomes were injected intravenously into a rat (upper position) at a dosage of 0.10 mmol/kg. The rat in the lower position served as control (e.g. no injection nor heating).

Figure 10 is the axial T₂-w SE image of the liver 7 minutes after liposome injection. At 15 minutes post injection, the liver was heated by focused ultrasound (Figure 11). Figures 12-13 are T₂-w SE images 16 and 21 minutes after initiation of heating, respectively.

After termination of heating, the measured temperature in the liver was 51°C.

The results indicate that the liver signal intensity increases substantially after heating as compared to the control liver.

Example 9

Preparation of pH-sensitive paramagnetic liposomes

Liposomes composed of DPPE/PA (4:1 mol/mol) containing GdDTPA-BMA were prepared by the thin film hydration method. The total lipid concentration was 25 mg/ml. Briefly, a chloroform/methanol (10:1) solution of the lipids was rotary evaporated to dryness and the resulting film was further dried under vacuum overnight. The lipids were hydrated with 250 mM GdDTPA-BMA in 0.05 M Tris-HCl buffer (pH = 8.4) at 75 °C. The liposomes were subjected to 3 freeze-thaw cycles in MeOH/CO₂(s). The liposomes were sized down by sequential extrusion (Lipex Extruder[®], Lipex Biomembranes Inc., Vancouver, Canada) through polycarbonate filters with various pore diameters. Untrapped metal chelate was removed by dialysis against isoosmotic glucose solution (pH=8.4).

Physicochemical properties

The mean hydrodynamic diameter of the liposomes was measured to 165 nm by photon correlation spectroscopy (ZetaSizer IV, Malvern Instruments Ltd., Malvern, England). The *in vitro* T₁-relaxation times of the paramagnetic liposomes were measured (0.235 T, Minispec PC-110b, Bruker GmbH, Rheinstetten, Germany) in different isoosmotic buffer solutions (0.05 M citrate-phosphate buffer and 0.05 M Tris-HCl buffer). The investigated pH range was 4-8.5. The buffered liposome dispersions were incubated at 37°C for 15 minutes. Table 11 shows the pH sensitivity of *in vitro* r₁-relaxivity for liposome encapsulated GdDTPA-BMA.

Table 11

pH dependency of the r (37 °C, 0.235 T) for liposomal GdDTPA-BMA

pH	r (s ⁻¹ mM ⁻¹)
3.91	1.32
4.30	1.26
4.70	1.31
5.15	1.17
5.59	1.10
5.95	1.03
6.40	1.00
6.71	0.50
7.33	0.32
7.69	0.29
8.02	0.28
8.34	0.29
8.54	0.31

Example 10

DyDTPA-BMA encapsulated within DPPC/DPPG liposomes

DPPC/DPPG (weight ratio; 95/5) liposomes were prepared by the thin film hydration method. The phospholipids (500mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. Liposomes were formed by hydrating the lipid film at 50°C with an aqueous solution (pH \approx 7) of 250 mM DyDTPA-BMA (sprodiamide, Nycomed Imaging AS, Oslo, Norway) (10ml). The liposomes were subjected to 3 freeze-thaw cycles and allowed to swell for one hour at 59°C. The liposome dispersion was extruded at 65°C through polycarbonate filters of various pore diameters. The liposome size (z-average) after extrusion was 153 nm. Untrapped DyDTPA-BMA was removed by dialysis against isoosmotic and isoprotic glucose solution. The temperature sensitivity of the MR contrast effect may be investigated.

Example 11GdDTPA-dextran encapsulated within DPPC/DPPG liposomes

DPPC/DPPG (weight ratio; 95/5) liposomes were prepared by the thin film hydration method. The phospholipids (500 mg) were dissolved in a chloroform/methanol mixture and the organic solution was evaporated to dryness under reduced pressure. The liposomes were formed by hydrating the lipid film at 48 C with an aqueous solution of 50 mM GdDTPA-dextran (MW 156 kD), whose synthesis is described in: *P Rongved et al., Carbohydr. Res., 287 (1996) 77-89*. The liposome dispersion was sonicated at 46 C using a sonicator tip. The liposome size (z-average) after sonication was 70 nm. Untrapped GdDTPA-dextran is removed by gel filtration or dialysis against isoosmotic and isoprotic glucose solution. The temperature sensitivity of the MR contrast effect may be investigated.

Example 12GdDTPA-BMA encapsulated within dibehenoyl-PC liposomes

Dibehenoyl-PC (22:0) (Table 1) liposomes may be prepared by the thin film hydration method. The phospholipids (500 mg) are dissolved in a chloroform/methanol mixture and the organic solution is evaporated to dryness under reduced pressure. Liposomes are formed by hydrating the lipid film at 80°C with an aqueous solution (pH \approx 7) of 250 mM GdDTPA-BMA (10 ml). The liposomes are subjected to 3 freeze-thaw cycles and allowed to swell for one and half-hours at 80°C. The liposome dispersion is extruded at 80°C through polycarbonate filters of various pore diameters. Untrapped GdDTPA-BMA is removed by gel filtration or dialysis against isoosmotic and isoprotic glucose solution. The temperature sensitivity of the MR contrast effect may be investigated.

Example 13

Superparamagnetic iron oxides (SPIOs) encapsulated within HPC/HPS liposomes

HPC/HPS (weight ratio; 90/10) liposomes were prepared by a modified thin film hydration method. Liposomes were formed by adding a homogeneous mixture of phospholipids (700 mg) to 10 ml of a pre-heated (55°C) aqueous dispersion of PEGylated SPIOs (6.10 mg iron/ml). The liposomes were allowed to swell for 30 minutes at 65°C. The liposome dispersion was extruded at 66°C through polycarbonate filters of various pore diameters. Untrapped SPIOs are removed by gel filtration or dialysis. The temperature sensitivity of the MR contrast effect may be investigated.

Example 14

Ultrasmall superparamagnetic iron oxides (USPIOs)
encapsulated within HPC/HPS liposomes

HPC/HPS (weight ratio; 90/10) liposomes were prepared by a modified thin film hydration method. Liposomes were formed by adding a homogeneous mixture of phospholipids (700 mg) to 10 ml of a pre-heated (70°C) aqueous dispersion of USPIOs (3.63 mg iron/ml). The liposomes were allowed to swell for 90 minutes at 70°C. The liposome dispersion was extruded at 70°C through polycarbonate filters of various pore diameters. Untrapped USPIOs are removed by gel filtration or dialysis. The temperature sensitivity of the MR contrast effect may be investigated.

Example 15

Superparamagnetic iron oxides (SPIOs) or ultrasmall-SPIOs encapsulated within pH-sensitive liposomes

SPIOs or USPIOs encapsulated within pH-sensitive

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liposomes may be prepared in a manner analogous to that used in Example 9. Untrapped superparamagnetic material is removed by gel filtration or dialysis. The pH-sensitivity of the MR contrast effect may be investigated.

Example 16

GdDTPA-BMA encapsulated within DSPC/DMPG/cholesterol liposomes

DSPC/DMPG/cholesterol liposomes (molar ratio; 49:5:20) were prepared by a modified thin film hydration method. Liposomes were formed by adding a freeze-dried mixture of phospholipids (60 g) to a pre-heated (59°C) aqueous solution (pH \approx 6.3) of 250 mM GdDTPA-BMA/300 mM sucrose/10mM phosphate (300 ml). The liposomes were allowed to swell for 30 minutes at 59°C. The liposome dispersion was homogenized and extruded at high pressure through polycarbonate filters with a pore size of 400 nm. Untrapped GdDTPA-BMA was removed by ultrafiltration with a 300 mM sucrose/10 mM phosphate solution. The liposome size (z-average) after ultrafiltration was 110 nm. Liposomes were also lyophilized (2ml per vial) and reconstituted by addition of 2 ml of deionized water. The liposome size (z-average) after reconstitution was 119 nm.

Table 12 summarizes the temperature sensitivity of the *in vitro* R_1 (0.235 T) for liposome encapsulated GdDTPA-BMA in a 300 mM sucrose/10 mM phosphate solution. The influence of lyophilization on the R_1 -temperature sensitivity of the reconstituted liposomes is also shown in Table 12.

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Table 12

	T Relaxation Time (ms)		
	35°C	40°C	55°C
Before lyophilisation	930	750	350
After lyophilisation	670	590	340

Example 17

Commercially available Gd compounds/Gd compounds in development phase encapsulated within temperature- or pH-sensitive liposomes

Liposomes containing the following contrast agents: GdBOPTA (Bracco spa, Italy), GdDTPA (Shering AG, Berlin), GdDOTA (Guerbet SA, Aulnay-sous-Bois), Gadomer (Shering AG, Berlin) MS-325 and protein bound MS-325 (Epix Medical Inc, USA) may be prepared in a manner analogous to that used in Examples 1-5, 9 and 12. Untrapped Gd compound is removed by gel filtration or dialysis. The temperature sensitivity of the MR contrast effect may be investigated.

Example 18

Temperature sensitivity of *in vitro* r, in blood for liposome encapsulated GdDTPA-BMA

DPPC/DPPG/DPPE-PEG-2000 and DPPC/DPPG liposomes containing GdDTPA-BMA were prepared in a manner analogous to that used in Examples 3 and 5, respectively. Table 13 summarizes the temperature evolution of the *in vitro* r (0.235 T) in rat blood for both liposome formulations.

Table 13

Temperature (°C)	r_1 ($s^{-1}mM^{-1}$) DPPC/DPPG 120 nm	r_1 ($s^{-1}mM^{-1}$) DPPC/DPPG/DPPE- PEG 121 nm
35	0.260	0.244
37	0.479	0.391
39	0.659	0.588
40	1.18	0.823
41	2.45	1.26
42	4.06	2.87
43	5.18	3.65
44	4.57	4.06

Example 19Pilot biodistribution and relaxometric studies of liposomal GdDTPA-BMA in male rats

a) Intramuscular injection

DPPC/DPPC liposomes containing GdDTPA-BMA (prepared in Example 18) were injected intramuscularly (im) into Sprague Dawley rats at a dosage of 20 :mol/kg.

Table 14 shows the T_1 relaxation times (37°C, 0.235 T) of excised tissues and blood one and three hours after im injection of DPPC/DPPG liposomes (n= 2x3). Table 15 shows the temperature response of the T_1 in muscle. All results are given as mean values.

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Table 14

Time post Injection (min)	T ₁ relaxation time (ms)		
0	500	833	248
60	451	950	249
180	440	833	243

Table 15

Time post Injection (min)	Muscle T ₁ (ms)	
0	500	340
60	451	341
180	440	383

Despite large interindividual variations, the results show the temperature dependence of the muscle T₁ after im administration of liposome encapsulated GdDTPA-BMA.

b) Intravenous injection

DPPC/DPPG/DPPE-PEG-2000 and DPPC/DPPG liposomes containing GdDTPA-BMA (prepared in *Example 18*) were injected intravenously (iv) into Sprague Dawley rats at a dosage of 100 :mol/kg.

Tables 16 and 17 show the T₁ relaxation times (37°C,

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0.235 T) of excised tissues and blood, 5 minutes (n=3), one (n=2) and three (n=3) hours after iv injection of DPPC/DPPG and DPPC/DPPG/DPPE-PEG-2000 liposomes, respectively. Also shown, is the Gd uptake in tissue, expressed as the percentage tissue uptake of the administered Gd dosage. Tables 18 and 19 summarize the temperature response of the blood T₁ after iv injection of DPPC/DPPG and DPPC/DPPG/DPPE-PEG-2000 liposomes, respectively. A more detailed investigation of the temperature response was performed in blood withdrawn one hour after iv administration (n=3) of DPPC/DPPG liposomes, as shown in Table 20. All results are given as mean values.

Table 16

Time post injection (min)	T ₁ relaxation time (ms)			
	<u>Tissue uptake (% of adm. Gd dosage)</u>			
	Liver	Blood	Spleen	Lungs
0	248	833	510	613
	-	-	-	-
5	212	533	357	547
	11.6	<u>60.0</u>	<u>1.9</u>	<u>0.88</u>
60	223	589	315	520
	<u>13.7</u>	<u>25.9</u>	<u>4.3</u>	<u>0.48</u>
180	227	823	353	557
	<u>7.9</u>	<u>1.3</u>	<u>2.0</u>	<u>0.06</u>

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Table 17

Time post injection (min)	T relaxation time (ms)			
	<u>Tissue uptake (% of adm. Gd dosage)</u>			
	Liver	Blood	Spleen	Lungs
0	248	833	510	613
	-	-	-	-
5	224	540	417	580
	<u>5.7</u>	<u>64.3</u>	<u>1.4</u>	<u>0.85</u>
180	202	500	298	563
	<u>8.8</u>	<u>27.7</u>	<u>6.5</u>	<u>0.56</u>

Table 18

Time post Injection (min)	Blood T _i (ms)	
	37°C	43°C
0	833	880
5	533	151
60	589	273
180	823	797

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Table 19

Time post Injection (min)	Blood T ₁ (ms)	
	37°C	43°C
0	833	880
60	540	135
180	500	244

Table 20

Blood T ₁ (ms)			
37°C	40°C	41°C	43°C
520	247	232	210

The results show the potential of both non-PEGylated and, especially, PEGylated liposomal GdDTPA-BMA as blood pool agents. The T₁-temperature sensitivity of the liposomes was also demonstrated in blood.

Example 20

In Vitro Imaging Studies with GdDTPA-BMA encapsulated within DSPC/DPPC/DPPG liposomes

DSPC/DPPC/DPPG liposomes containing GdDTPA-BMA were prepared analogously to Example 2. The liposome size (z-average) was 129 nm. MR imaging was performed at 2.0 T (Bruker Medspec) on a concentric spherical phantom in which the inner chamber contained liposomal GdDTPA-BMA diluted with an isotonic medium composed of glucose and 6.25% polyvinylpyrrolidone (conc. \approx 0.8 mM Gd), whilst the outer compartment was filled with saline. Microwave heating was performed at 434 MHz with a linear radio

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The temperature evolution of the measured R_1 for liposomal GdDTPA-BMA is summarized in Figure 14. A linear correlation was obtained between R_1 and temperature in the "transition region" 40.4-43.7°C (regression coefficient of 0.995). Figure 15 shows selected T_1 -W GE images of the phantom (a) before heating, (b) during heating; signal intensity distribution observed within liposome sample, and (c) after heating; homogeneous signal intensity distribution. Figure 16 shows the corresponding T_1 -maps at the same time points as for Figure 15. By use of the linear correlation between R_1 and temperature, a corresponding temperature map could be derived from the T_1 -map at timepoint (b), as seen on Figure 17. The temperature map demonstrates the thermosensitivity of liposomal GdDTPA-BMA. (NB The temperature scale is only valid for the inner chamber containing liposomes).

Example 21In vitro MR imaging studies with GdDTPA-BMA encapsulated within DPPC/DPPG liposomes - Determination of Gd concentration.

A static *in vitro* phantom, composed of twelve glass vials (10 mm dia.) placed in a rectangular plastic container, was used for this study. The plastic container was filled with a viscous isotonic medium composed of glucose/25% (w/w) polyvinyl-pyrrolidone (PVP) and doped with GdDTPA-BMA to give a T_1 of about 430 ms at 1.5 T. Three of the vials contained a marker solution with a known T_1 value (about 630 ms). The remaining nine vials were filled with DPPC/DPPG-based GdDTPA-BMA liposomes (prepared in Example 18) dispersed in varying amounts of isotonic 10% PVP/glucose solution. The Gd concentration [Gd] in the liposome samples ranged from 0 to 5.2 mM Gd as determined by inductively coupled plasma atomic emission spectrophotometry. The phantom was imaged at room temperature in a quadrature knee coil at 1.5 T on a Philips NT system. The following imaging sequences were used:

1. T_1 -FFE (spoiled gradient echo) TR/TE/flip: 15ms/2ms/30°.
2. TMIX (quantitative T_1/T_2 sequence)
3. Dual TE FFE (T_2^* mapping) (TE1/TE2: 4ms/50 ms)

All three sequences were repeated after heating of the phantom, the latter achieved by placing the phantom in a warm (>60°C) water bath. The temporal effect of heating was not investigated, only the end-effect (i.e. $T \gg T_1$).

A linear correlation was obtained between [Gd] and matrix corrected R_1 (ΔR_1) prior to heating (measured from TMIX sequence), with a regression coefficient of 0.996. The calculated liposomal r_1 was $0.11 \text{ mM}^{-1}\text{s}^{-1}$.

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Analogously, a liposomal r of $0.55 \text{ mM}^{-1} \text{ s}^{-1}$ was determined from the R vs $[\text{Gd}]$ curve (regression coefficient = 0.997); the r/r ratio being equal to 5. After liposome heating, the r and r were 3.23 and 3.75, respectively, giving an r/r ratio of 1.16.

Figure 18 shows, prior to heating, a linear correlation between $[Gd]$ and the ratio of the signal intensities of liposome sample and PVP gel (SI_{lip}/SI_{PVP}) using the T_1 -FFE sequence. Figure 19 shows the plot of the $|R_1|/|R_2|$ ratio vs the $[Gd]/[Gd^{free}]$ ratio; here $[Gd^{free}] = 5.2$ mM. The results demonstrated that prior to heating, the $|R_1|$ ratio accurately reflected the $[Gd]$ ratio. Similar results were also obtained when the $|R_2|$ ratio was employed. The findings suggest that the T_1 - (and T_2 -) effect of liposome encapsulated GdDTPA-BMA prior to heating is significant enough to enable a relative assessment of liposomal Gd concentration.

Example 22

In vitro MR imaging studies with GdDTPA-BMA encapsulated within DPPE/PA liposomes

DPPE/PA liposomes containing GdDTPA-BMA were prepared analogously to Example 9. The mean hydrodynamic diameter of the liposomes was measured to 158 nm by photon correlation spectroscopy (Malvern PS/MW 4700, Malvern Instruments Ltd., Malvern, England). An *in vitro* phantom, composed of thirteen glass vials (11 mm diameter) placed in a circular glass reactor, was used for this study. The glass reactor was filled with an agar gel (2 % w/v) doped with GdDTPA-BMA to give a T_1 of about 900 ms at 1.5 T. The glass vials were filled with isoosmotic buffer solutions with pHs ranging from 4.8 to 8.2. The phantom was constantly held at a temperature of 37°C by circulating heated water through the shell of the reactor with a circulating water pump. Liposomes were added successively to each vial with a

	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	2027	2028	2029	2030	2031	2032	2033	2034	2035	2036	2037	2038	2039	2040	2041	2042	2043	2044	2045	2046	2047	2048	2049	2050	2051	2052	2053	2054	2055	2056	2057	2058	2059	2060	2061	2062	2063	2064	2065	2066	2067	2068	2069	2070	2071	2072	2073	2074	2075	2076	2077	2078	2079	2080	2081	2082	2083	2084	2085	2086	2087	2088	2089	2090	2091	2092	2093	2094	2095	2096	2097	2098	2099	2100	2101	2102	2103	2104	2105	2106	2107	2108	2109	2110	2111	2112	2113	2114	2115	2116	2117	2118	2119	2120	2121	2122	2123	2124	2125	2126	2127	2128	2129	2130	2131	2132	2133	2134	2135	2136	2137	2138	2139	2140	2141	2142	2143	2144	2145	2146	2147	2148	2149	2150	2151	2152	2153	2154	2155	2156	2157	2158	2159	2160	2161	2162	2163	2164	2165	2166	2167	2168	2169	2170	2171	2172	2173	2174	2175	2176	2177	2178	2179	2180	2181	2182	2183	2184	2185	2186	2187	2188	2189	2190	2191	2192	2193	2194	2195	2196	2197	2198	2199	2200	2201	2202	2203	2204	2205	2206	2207	2208	2209	2210	2211	2212	2213	2214	2215	2216	2217	2218	2219	2220	2221	2222	2223	2224	2225	2226	2227	2228	2229	2230	2231	2232	2233	2234	2235	2236	2237	2238	2239	2240	2241	2242	2243	2244	2245	2246	2247	2248	2249	2250	2251	2252	2253	2254	2255	2256	2257	2258	2259	2260	2261	2262	2263	2264	2265	2266	2267	2268	2269	2270	2271	2272	2273	2274	2275	2276	2277	2278	2279	2280	2281	2282	2283	2284	2285	2286	2287	2288	2289	2290	2291	2292	2293	2294	2295	2296	2297	2298	2299	2300	2301	2302	2303	2304	2305	2306	2307	2308	2309	2310	2311	2312	2313	2314	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	2327	2328	2329	2330	2331	2332	2333	2334	2335	2336	2337	2338	2339	2340	2341	2342	2343	2344	2345	2346	2347	2348	2349	2350	2351	2352	2353	2354	2355	2356	2357	2358	2359	2360	2361	2362	2363	2364	2365	2366	2367	2368	2369	2370	2371	2372	2373	2374	2375	2376	2377	2378	2379	2380	2381	2382	2383	2384	2385	2386	2387	2388	2389	2390	2391	2392	2393	2394	2395	2396	2397	2398	2399	2400	2401	2402	2403	2404	2405	2406	2407	2408	2409	2410	2411	2412	2413	2414	2415	2416	2417	2418	2419	2420	2421	2422	2
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time interval of 1 minute. The imaging was started 25 minutes after addition of liposomes to the first vial. The phantom was imaged at 1.5 T on a Philips NT system. The following imaging parameters were used: sequence: MIX-TSE; TR (ms): 800.0; TE (ms): 12.5; TI (ms): 500.0; flip (deg): 90; slice thickness (mm): 7.0; FoV (freq*phase, mm): 230.0*230.0. The scan cycles were repeated every minute for 20 minutes. *Figure 20* shows the phantom 25 minutes after addition of liposomes to the first vial. The signal intensity increases with decreasing pH.

Example 23.

Particles from polymer made from ethylidene bis(16-hydroxyhexadecanoate) and adipoyl chloride

Air filled particles of the polymer ethylidene bis(16-hydroxyhexadecanoate) and adipoyl chloride were made as described in Example 3f of WO 96/07434.

Change of gas

The dry powder was exposed to 20 mmHg vacuum for ca. 15 minutes, followed by inlet of perfluorobutane gas. The powder of polymer particles containing perfluorobutane gas were then redispersed to 10 mg/ml dry material in MilliQ water by shaking on a laboratory shaker for 12-16 hours. Examination by light microscopy indicated formation of a particle dispersion with irregular shaped particles. The particles floated readily, as expected for gas-containing particles.

Heating of the polymer particles

10 ml of the polymer dispersion was heated on a water bath to 65°C in one minute while using magnet stirring. At this temperature the polymer melted. Microscopic evaluation indicated that the irregular particles changed to spherical, smooth particles,

[illegible]

indicating that the polymer capsule melted.

Characterization

The acoustic effect of the suspension prepared above was obtained by measuring the ultrasonic transmission through a dispersion in an aqueous carrier liquid, using a 3.5 MHz broadband transducer in a pulse-reflection technique. The aqueous carrier liquid was used as reference. Table 21 contains the observed acoustic attenuation compared to non-heated particle dispersion at the same concentrations, indicating that heat treatment removes most of the acoustic attenuation.

The gas content was measured by density measurements before and after destruction of the microcapsules using high-energetic ultrasound. The results show that the gas content is conserved. The results show that by melting the polymer capsules, the gas filled microcapsules become almost invisible to ultrasound, probably due to the stiff polymer shell now surrounding the gas phase.

Table 21. Characteristics of perfluorobutane containing polymer particles treated by heating the dispersions to 65°C.

Description	Gas content [% v/v (rel. sample vol.)]	Acoustic attenuation [dB/cm]
Reference	1.398	6.42
65°C, 1 min	1.227	0.63

Example 24

The particles from example 23 above are studied by acoustic characterisation in vitro by measuring the ultrasonic transmission through a dispersion in an aqueous carrier liquid, using a 3.5 MHz broadband

transducer in a pulse-reflection technique. The aqueous carrier liquid is used as reference. The acoustic characterisation is started at room temperature where the acoustic attenuation is low as shown in Table 21 above. The temperature is raised and acoustic characterisations are done at different temperature intervals. When the temperature passes 48.6°C, the melting point of the polymer (see example 23 above), the acoustic attenuation increases sharply, indicating a temperature sensitive contrast agent. A similar experiment could be done using a polymer with melting point around 37-40°C, and hence closer to body temperature.

Example 25

A spatula edge of micronised kaolin is added to 2 ml perfluorodimethylcyclobutane (b.p. 45°C) and dispersed using 0.2 ml FluoradTM FC-171 surfactant. A milky white dispersion is obtained by vigorously stirring.

1 ml of a dispersion of 1,2-distearoyl-phosphatidyl glycerol and (0,5 mg/ml) and distearoylphosphatidyl-choline (4,5 mg/ml) in purified water is placed in a 2 ml vial to which is added 100 µl of the kaolin in perfluorodimethylcyclobutane dispersion described above. The vial is closed and then shaken for 75 seconds using an Espe CapMix[®] to yield a kaolin in perfluorodimethylcyclobutane in water emulsion.

An acoustic apparatus is mounted in which the acoustic effect of the suspension prepared above can be obtained by measuring the ultrasonic transmission through a dispersion in an aqueous carrier liquid, using a 3.5 MHz broadband transducer in a pulse-reflection technique. The aqueous carrier liquid can be used as reference. The sample is injected in a thermostatted cell, where an overpressure or an underpressure can be

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applied by a pump.

The emulsion is transformed to the acoustic cell and diluted with 50 ml water, keeping the temperature constant at 37°C. The first measurement is done at atmospheric pressure showing weak acoustic attenuation due to the presence of liquid emulsion droplets and no gas bubbles in the cell. The pressure is then gradually reduced at intervals, and the acoustic measurement is done at each interval. When the pressure reach 580 mmHg (i.e. 180 mmHg below atmospheric pressure), the acoustic attenuation increases sharply and significantly, demonstrating that the microdroplets now boil and turn to acoustic effective microbubbles.

This experiment demonstrate how an emulsion with a disperse phase of boiling point slightly above body temperature can be used to map underpressure in vivo, for instance the underpressure which will occur below an embolisation of a blood vessel.

Example 26

GdDTPA-BMA encapsulated within Mg^{2+} sensitive liposomes

Beef-heart cardiolipin - cesium salt, dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol - potassium salt (DPPG) are added to a round bottom flask in 40/55/5 mol ratio (totally 500 mg) and dissolved using chloroform. The chloroform is removed by evaporation under reduced pressure using a rotavapor. Liposomes are formed by hydrating the lipid film with a pre-heated (52°C) aqueous solution of 250 mM GdDTPA-BMA (10 ml). The liposomes are subjected to 3 freeze-thaw cycles and allowed to swell for one and half-hour at 55°C. The liposome dispersion is extruded at 62°C through polycarbonate filters of various pore diameters. Untrapped GdDTPA-BMA is removed by dialysis against isoosmotic and isoprotic glucose solution.

Country	Year	Population (millions)	Urban population (millions)	Urban population (%)	Population density (per sq km)	Urban population density (per sq km)	Population growth rate (%)	Urban population growth rate (%)	Population growth rate (%)	Urban population growth rate (%)	Population growth rate (%)	Urban population growth rate (%)
Algeria	1980	10.0	4.0	40.0	100	250	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	1985	10.5	4.5	42.9	105	262	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	1990	11.0	5.0	45.5	110	275	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	1995	11.5	5.5	47.8	115	288	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2000	12.0	6.0	50.0	120	300	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2005	12.5	6.5	52.0	125	312	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2010	13.0	7.0	53.8	130	325	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2015	13.5	7.5	55.6	135	338	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2020	14.0	8.0	57.1	140	350	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2025	14.5	8.5	58.6	145	362	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2030	15.0	9.0	60.0	150	375	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2035	15.5	9.5	61.3	155	388	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2040	16.0	10.0	62.5	160	400	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2045	16.5	10.5	63.6	165	412	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2050	17.0	11.0	64.7	170	425	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2055	17.5	11.5	65.7	175	438	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2060	18.0	12.0	66.7	180	450	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2065	18.5	12.5	67.6	185	462	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2070	19.0	13.0	68.4	190	475	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2075	19.5	13.5	69.2	195	488	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2080	20.0	14.0	70.0	200	500	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2085	20.5	14.5	70.7	205	512	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2090	21.0	15.0	71.4	210	525	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2095	21.5	15.5	72.1	215	538	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2100	22.0	16.0	72.7	220	550	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2105	22.5	16.5	73.3	225	562	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2110	23.0	17.0	73.9	230	575	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2115	23.5	17.5	74.5	235	588	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2120	24.0	18.0	75.0	240	600	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2125	24.5	18.5	75.5	245	612	1.5	2.5	1.5	2.5	1.5	2.5
Algeria	2130	25.0	19.0	76.0	250	625	1.5	2.5	1.5	2.5	1.5	

10 **11** **12** **13** **14** **15** **16** **17** **18** **19** **20** **21** **22** **23** **24** **25** **26** **27** **28** **29** **30** **31** **32** **33** **34** **35** **36** **37** **38** **39** **40** **41** **42** **43** **44** **45** **46** **47** **48** **49** **50** **51** **52** **53** **54** **55** **56** **57** **58** **59** **60** **61** **62** **63** **64** **65** **66** **67** **68** **69** **70** **71** **72** **73** **74** **75** **76** **77** **78** **79** **80** **81** **82** **83** **84** **85** **86** **87** **88** **89** **90** **91** **92** **93** **94** **95** **96** **97** **98** **99** **100** **101** **102** **103** **104** **105** **106** **107** **108** **109** **110** **111** **112** **113** **114** **115** **116** **117** **118** **119** **120** **121** **122** **123** **124** **125** **126** **127** **128** **129** **130** **131** **132** **133** **134** **135** **136** **137** **138** **139** **140** **141** **142** **143** **144** **145** **146** **147** **148** **149** **150** **151** **152** **153** **154** **155** **156** **157** **158** **159** **160** **161** **162** **163** **164** **165** **166** **167** **168** **169** **170** **171** **172** **173** **174** **175** **176** **177** **178** **179** **180** **181** **182** **183** **184** **185** **186** **187** **188** **189** **190** **191** **192** **193** **194** **195** **196** **197** **198** **199** **200** **201** **202** **203** **204** **205** **206** **207** **208** **209** **210** **211** **212** **213** **214** **215** **216** **217** **218** **219** **220** **221** **222** **223** **224** **225** **226** **227** **228** **229** **230** **231** **232** **233** **234** **235** **236** **237** **238** **239** **240** **241** **242** **243** **244** **245** **246** **247** **248** **249** **250** **251** **252** **253** **254** **255** **256** **257** **258** **259** **260** **261** **262** **263** **264** **265** **266** **267** **268** **269** **270** **271** **272** **273** **274** **275** **276** **277** **278** **279** **280** **281** **282** **283** **284** **285** **286** **287** **288** **289** **290** **291** **292** **293** **294** **295** **296** **297** **298** **299** **300** **301** **302** **303** **304** **305** **306** **307** **308** **309** **310** **311** **312** **313** **314** **315** **316** **317** **318** **319** **320** **321** **322** **323** **324** **325** **326** **327** **328** **329** **330** **331** **332** **333** **334** **335** **336** **337** **338** **339** **340** **341** **342** **343** **344** **345** **346** **347** **348** **349** **350** **351** **352** **353** **354** **355** **356** **357** **358** **359** **360** **361** **362** **363** **364** **365** **366** **367** **368** **369** **370** **371** **372** **373** **374** **375** **376** **377** **378** **379** **380** **381** **382** **383** **384** **385** **386** **387** **388** **389** **390** **391** **392** **393** **394** **395** **396** **397** **398** **399** **400** **401** **402** **403** **404** **405** **406** **407** **408** **409** **410** **411** **412** **413** **414** **415** **416** **417** **418** **419** **420** **421** **422** **423** **424** **425** **426** **427** **428** **429** **430** **431** **432** **433** **434** **435** **436** **437** **438** **439** **440** **441** **442** **443** **444** **445** **446** **447** **448** **449** **450** **451** **452** **453** **454** **455** **456** **457** **458** **459** **460** **461** **462** **463** **464** **465** **466** **467** **468** **469** **470** **471** **472** **473** **474**

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of 92 mg/ml. 2 ml portions of the resulting dispersion were filled into 10 ml flat-bottomed vials specially designed for lyophilisation, and the contents were lyophilised to give a white porous cake. The lyophilisation chamber was then filled with perfluorobutane and the vials were sealed. Prior to use, water was added to a vial and the contents were gently hand-shaken for several seconds to give a perfluorobutane microbubble dispersion.

Microscopic investigation

One drop of the microbubble dispersion was placed on an object glass for microscopy investigation. The sample was covered with a cover glass and placed under a microscope. Droplets of a 50 mg/ml calcium chloride solution in water were added to the edge of the cover glass so that the solution penetrated into the microbubble dispersion. The behaviour of the microbubble dispersion as the calcium chloride solution front moved was recorded on videotape. Microbubble aggregates with larger dimensions than the initial microbubbles were observed to form, demonstrating that microbubbles with potential to have different ultrasound properties were generated.

Example 29

TcDTPA encapsulated within DPPC/DPPG liposomes

TcDTPA is prepared from sodium pertechnetate and a commercial kit containing SnCl_2 and DTPA. TcDTPA is encapsulated in liposomes similar to Example 10 above. The product is a temperature sensitive contrast agent for scintigraphic studies.

Example 30Gadolinium DTPA labelled starch microspheres

Gadolinium DTPA starch particles were prepared according to P. Rongved et al. in Carbohydrate Research 214 (1991) 325-330 substrate 9 to 12. The particles were suspended in 0.9 % NaCl solution before administration. The product can be used to diagnose diseases related to abnormal enzyme activity (α -amylase and esterase); for example.

Example 31Iodixanol-containing liposomes

A diagnostic composition comprising:

Iodixanol (total amount)	400 mg/ml
Iodine encapsulated	80 mg/ml
Sorbitol	20 mg/ml
Trometamol (TRIS)	0.097 mg/ml
EDTANa ₂ Ca	0.1 mg/ml
Hydrogenated phosphatidylcholine	51.2 mg/ml
Hydrogenated phosphatidylserine	5.1 mg/ml
Water for injection ad	1 ml (approx 0.9 ml)

was prepared by dissolving the phospholipid in chloroform/methanol/water (4:1:0.025, volume) and evaporating the solvent (rotary evaporation). An isotonic solution of iodixanol and sorbitol was made and heated to 60-70°C and this temperature was maintained during the remainder of the process. The phospholipid mixture was added with stirring, and the liposomes were formed. To control the size of the liposomes the preparation was homogenized (Rotor/Stator homogenizer). The liposomes were then extruded through 7 polycarbonate filters placed in series (pore diameter 1 μ m). The

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product was diluted with an isotonic solution of iodixanol and sorbitol, and trometamol and EDTA were added. The product was filled into glass vials and autoclaved (121°C, 15 minutes). Tc = 49 °C.

The product can be used to monitor temperature during hyperthermia treatment with focused ultrasound.

Example 32

Gas containing microbubbles of DPPC/PPG/DPPE-PEG "doped"
with vector with affinity for angiogenesis

Various products can be prepared using the technology described in WO 98/18500. The products can be used as tumor specific markers for hyperthermia treatment.

[illegible]